Biochimica et Biophysica Acta, 1103 (1992) 13–24

BBAMEM 75484

Probing the active site of the reconstituted aspartate/glutamate carrier from bovine heart mitochondria: carbodiimide-catalyzed acylation of a functional lysine residue

Thomas Dierks *, Reiner Stappen, Angelika Salentin and Reinhard Krämer

Institut für Biotechnologie, Forschungszentrum Jülich, Jülich (Germany)

(Received 22 August 1991)

Key words: Mitochondrion; Aspartate/glutamate carrier; Reconstitution; Protein modification; Carbodiimide; Carrier-mediated transport

Upon modification of the reconstituted aspartate/glutamate carrier by various amino acid-reactive chemicals a functional lysine residue at the exofacial binding site was identified. The inactivation of transport function by the lysine-specific reagents pyridoxal phosphate (PLP, IC_{50} 400 μM) and 4-acetamido-4'-isothiocyanostilbene-2,2'-disulfonate (SITS, IC_{50} 300 μM) could specifically be suppressed by the substrates aspartate and glutamate; a 50% substrate protection was observed at half-saturation of the external binding site. The same held true for 1-ethyl-3-(3-dimethylaminopropyl)carbodiimide (EDC, IC_{50} 500 μM) and diethyl pyrocarbonate (DEPC, IC_{50} 20 μM), two reagents known to modify carboxylic or histidinyl side-chains, respectively. EDC, however, turned out to catalyze an acylation of the active site lysine by activating carboxyls that had to be present in the incubation medium. This special mechanism, which was proven by protein labelling using EDC/[^{14}C]succinate, necessitates a lysine side-chain of high reactivity and low pK, since the modification had to occur at pH ≤ 6.5, i.e. not too far from the pK of the carboxyl to be activated. All reagents applied, additionally including 4,4'-diisothiocyanostilbene-2,2'-disulfonate (DIDS, IC_{50} 10 μM), were effective at this pH. Competition experiments revealed interaction of EDC, PLP, SITS and probably DIDS at the same active site lysine. For DEPC a lysine modification could not be ruled out. Yet, a model comprising a histidine juxtaposed to the lysine seems to be appropriate.

Introduction

Among the various specialized transport proteins found in the inner mitochondrial membrane (for reviews see Refs. 1–3) the Asp/Glu carrier in functional respect is one of the best characterized systems. The isolated carrier [4] was reconstituted with right-side out orientation into liposomal membranes [5,6], thus allowing to investigate the kinetic mechanism of Asp/Glu antiport under defined conditions [7,8]. We found an antiport mechanism of the sequential type implicating the occurence of a ternary complex of the protein with two substrate ligands during translocation. Two independent substrate binding sites, one on each membrane side, are involved in the formation of this catalytic complex, as had to be concluded from the kinetic patterns obtained with proteoliposomes and, in parallel, also with intact mitochondria [6,7]. This so-called rapid-equilibrium random mechanism turned out to be valid also for the oxoglutarate carrier as was shown both in mitochondria [9] and in proteoliposomes [10]. Considering furthermore recent data in favor of such a mechanism for the ADP/ATP carrier [11] it becomes

* Present address: Zentrum Biochemie/Abteilung Biochemie II, Universität Göttingen, Gosslerstrasse 12d, D-3400 Göttingen, Germany.

Abbreviations: Asp/Glu carrier, aspartate/glutamate carrier; DEPC, diethyl pyrocarbonate; DIDS, 4,4'-diisothiocyanostilbene-2,2'-disulfonate; DNDS, 4,4'-dinitrostilbene-2,2'-disulfonate; DTE, 1,4-dithioerythritol; EDC, 1-ethyl-3-(3-dimethylaminopropyl)carbodiimide; GME, glycine methyl ester; IC_{50}, inhibitor concentration leading to half-maximum inhibition; Mops, 3-(*N*-morpholino)propanesulfonic acid; PLP, pyridoxal 5'-phosphate; $S_{0.5}$, substrate concentration leading to half-maximum substrate protection; SDS, sodium dodecylsulfate; SITS, 4-acetamido-4'-isothiocyanostilbene-2,2'-disulfonate.

Correspondence: R. Krämer, Institut für Biotechnologie 1, Forschungszentrum Jülich, P.O. Box 1913, D-5170 Jülich, Germany.

evident that the similarity of the members of the structural family of mitochondrial carrier proteins [12–14] is expressed also by common functional principles [6,10].

The elucidation of the molecular translocation mechanism, nevertheless, is far from being solved for any biological transport system due to the lack of the 3D-structure of carrier proteins. Since with membrane proteins only in exceptional cases spectroscopic or diffraction studies can be carried out, the chemical modification, besides mutagenesis, is the method of choice to reveal elementary structural aspects underlying translocation. Such structure-function relationships already were described previously for the reconstituted Asp/Glu carrier (and ADP/ATP carrier) [15,16]. By using cysteine-modifying reagents a basic change in the transport function could be induced transforming the obligatory antiport carriers reversibly into unidirectional transport systems (efflux carriers). For antiport-efflux interconversion two reactive cysteines had to be modified by mercury compounds. The pore-like characteristics observed for the efflux activity were taken as evidence for a protein-immanent channel structure as a prerequisite not only for efflux but also for the physiological substrate translocation (antiport) [16].

In order to gain further insight into the structural basis for this dual function in the present paper we examined the influence of additional amino acid-reactive modifiers on the antiport and as well on the SH-induced efflux state of the Asp/Glu carrier. By elucidating the chemical reaction mechanisms of the applied reagents and the mutual interaction of reagent and substrate with the carrier protein informations could be obtained about functional amino acid side-chains along the transport pathway and/or at the active binding site of the carrier. With the intention to selectively label the affected protein further experiments were carried out using radioactive reagents.

Materials and Methods

Materials and their sources

AMPLIFY, L-[U-^{14}C]aspartate, [8-^{14}C]ATP, L-[U-^{14}C]glutamate, ^{14}C-methylated Rainbow protein molecular weight markers and [1,4-^{14}C]succinate were obtained from Amersham-Buchler. Sigma supplied the following chemicals: DL-α-aminoadipic acid, carboxyatractyloside, DEPC, [*carbonyl*-^{14}C]DEPC (3.8 mCi/mmol), DTE, 5,5′-dithiobis(2-nitrobenzoic acid), EDC, fluorescein-5-isothiocyanate, GME, lysine methyl ester, mersalyl acid, Mops, *p*-(chloromercuri)benzoate and turkey egg-yolk phospholipid. Amberlite XAD-2, 4-bromophenacyl bromide, Dowex 1-X8, phenylisothiocyanate and SITS were purchased from Fluka, hydroxyapatite (Bio-Gel HTP) from Bio-Rad, Sephadex from Pharmacia, PLP from Merck and DIDS from Pierce. Bongkrekic acid was isolated by Prof. Berends (Delft). DNDS was a gift of Dr. L. Zaki (Frankfurt). All further chemicals were of analytical grade.

Preparation of proteoliposomes

The preparation of the Asp/Glu carrier from bovine heart mitochondria was described previously [4]. The protein fraction obtained after hydroxyapatite centrifugation chromatography and desalting on Sephadex G-25 was used for reconstitution. This fraction contained both Asp/Glu and ADP/ATP carrier protein.

The protein was incorporated into liposomes by hydrophobic chromatography of mixed micelles on Amberlite beads in a recycling procedure [17]. The exact reconstitution conditions were described by Dierks and Krämer [5]. As internal substrates the proteoliposomes contained 16 mM aspartate or, for substrate protection experiments, 8 mM aspartate/8 mM glutamate, and, for comparative investigations of the ADP/ATP carrier, additionally 10 mM ATP.

Chemical modification of the carrier protein

The various modifying reagents applied, except $HgCl_2$, mersalyl, 5,5′-dithiobis(2-nitrobenzoate) and *p*-(chloromercuri)benzoate, were prepared freshly for each experiment. As solvents were used: absolute ethanol (DEPC), 50% ethanol (SITS, DIDS), acetonitrile (4-bromophenacyl bromide), dimethyl sulfoxide (fluorescein 5-isothiocyanate), 50 mM phosphate buffer (pH 7) (5,5′-dithiobis(2-nitrobenzoic acid)), the respective gel filtration buffer (100 mM Mops, 50–80 mM sucrose, pH 6.5) (PLP) or water.

The modification of the Asp/Glu carrier (and ADP/ATP carrier) in all cases was carried out by incubating reconstituted protein with these reagents at room temperature and at pH 6.5 (EDC: pH 6.2). Previously, however, the external substrate was removed by gel filtration of proteoliposomes on Sephadex G-75 (see below). In those experiments concerning substrate protection the substrate (or the protector) was added in the appropriate concentration prior to the reagent. This also applies to the carbonic acids needed for modification by EDC (see Results). The reagents always were added from a 50-fold concentrated stock solution, while adding the respective solvent to the controls (final concentration $\leq 2\%$). In competition experiments the second reagent was added after a short preincubation with the first reagent. The exact conditions are given in the legends of the respective experiments. The modification reactions usually were stopped by adding GME (EDC), DTE (SITS, DIDS) or histidine (DEPC) or, in any case, by finally removing the reagents on a second gel filtration column.

For testing the influence of the different reagents on efflux activity the proteoliposomes had to be loaded with [^{14}C]aspartate (or [^{14}C]ATP) (see below) before incubating them with the reagent in the presence of

the radioactivity (approx. 0.5 μM aspartate or ATP, respectively). In the corresponding controls the effect on antiport activity was determined by the backward exchange method which is experimentally related to the efflux measurements (see below).

Transport measurement

The reconstituted transport activities (antiport and efflux) of the Asp/Glu and ADP/ATP carrier were determined by measuring the flux of ^{14}C-labelled substrate, as was described previoulsy [15]. For monitoring the antiport activity two different techniques were applied. In forward exchange experiments (uptake of label) the assay was started by the addition of labelled aspartate (or ATP, respectively) to proteoliposomes containing unlabelled countersubstrate inside. In backward exchange experiments (export of label) unlabelled substrate was added to proteoliposomes containing prelabelled internal substrate pools. In both modes of antiport measurement the reaction was stopped using 40 mM pyridoxal phosphate (or 33 μM carboxyatractyloside/5 μM bongkrekic acid, respectively).

The procedure for measuring unidirectional transport (efflux) activity resembled the backward exchange method. The export of label, however, was started by adding 100 μM mersalyl (in the absence of external substrate) and stopped by adding 5 mM dithioerythritol/40 mM pyridoxal phosphate, as was done both in the case of aspartate and ATP efflux assays. For prelabelling the internal substrate pool in efflux or backward exchange experiments, proteoliposomes, after the first gel filtration, were loaded with labelled substrate by antiport activity in the forward exchange mode (8 min) adding 0.5 μM aspartate (or ATP) of high specific radioactivity.

The transport activity (forward exchange, backward exchange or efflux) usually was measured after removal of all reagents, i.e. after the second gel filtration. Only when determining the total inhibition caused by PLP the measurements were carried out in the presence of the reagent (see Results). Further experimental details concerning gel filtration chromatography, removal of external radioactivity on anion exchange material and evaluation of transport activities by computer fitting are given in Refs. 15 and 5.

Protein labelling

The EDC-mediated ^{14}C-succinylation was carried out according to the above instructions using, however, 0.1–0.5 mM [^{14}C]succinate as a carboxyl component (specific radioactivity $\leq$ 100 dpm/pmol). More detailed incubation conditions are given in the legend of Fig. 3. The incorporation of radioactivity into the proteoliposomes was checked by fractionating the eluate of the gel filtration column. The labelling of the protein could be quantitated during preparation of samples for electrophoresis by precipitating the protein with deoxycholate and trichloroacetic acid [15] and extracting detergent and lipid with organic solvents [15]. Normally $\leq$ 5 μg of protein containing $\leq$ 200 pmol of label were applied on a SDS-polyacrylamide gel. The gels contained 10% acrylamide/0.3% bisacrylamide and were prepared, run and stained according to the protocol of Schägger and Von Jagow [18]. For fluorographic detection of radioactivity the stained gels were treated with AMPLIFY (Amersham) for 30 min and dried on filter paper in vacuo (70°C, 90 min). The fluorographic exposure of a non pre-flashed X-ray film (Fuji RX) took 1–6 weeks at −70°C.

For labelling with [^{14}C]DEPC (8 dpm/pmol) much higher concentrations ($\approx$ 1 mM) had to be chosen than applied for inactivation studies in order to achieve sufficient incorporation of label. Due to the incomplete blocking efficiency of added histidine at this high DEPC concentration (see Results) the final gel filtration passage had to be as fast as possible to stop the modification reaction. During preparation of protein samples and electrophoresis high losses of label were observed, as is explained by the low stability of the modification product [19].

Results

Modification by pyridoxal phosphate

It has been shown several years ago [5,20] that the Asp/Glu carrier, like other mitochondrial anion carriers, is inhibited by pyridoxal phosphate (PLP). This reagent modifies proteins specifically at lysine residues [21,22]. Due to this high specificity (see also references in Ref. 23) PLP in our investigations served as a reference reagent, which in competition experiments was applied to test whether other reagents also act on lysine residue(s) of the protein. For this purpose another favorable property of PLP was taken advantage of. Since the condensation reaction of PLP and lysine side-chains under Schiff base formation is reversible [22,24], particularly at non-alkaline pH, bound reagent may be removed again by passing the reconstituted protein through a gel filtration column. As shown in Table I the inactivation of the Asp/Glu carrier could in fact be reverted to a large extent by this method, i.e. 40–60% of the activity was restored even after treatment of proteoliposomes with 20 mM PLP, a concentration high above the K_i (see below). The degree of reversibility depends on the protein under study [25].

We found that the Asp/Glu carrier was protected from inhibition by the presence of external substrate. Since PLP acted as a reversibly binding ligand, competition experiments between substrate and PLP could be carried out (cf. Ref. 26). Fig. 1 demonstrates that saturating concentrations of aspartate (> 300 μM)

TABLE I

Inactivation of the reconstituted Asp / Glu carrier after chemical modification by various reagents

Relative antiport and efflux activities are compared. In addition, the effects of the reagents on the coreconstituted ADP/ATP carrier are given. Proteoliposomes, preloaded with labelled substrate (see Methods), were incubated with the respective reagent at the indicated concentration for 10 min (PLP), 7.5 min (EDC, inactivation of antiport), 15 min (EDC, inactivation of efflux; DEPC, SITS) or 5 min (DIDS). Unless otherwise indicated (see footnote), non-reacted reagent was removed by gel filtration of liposomes prior to the activity assay. Backward exchange and efflux were initiated by adding 1 mM external substrate (aspartate or ATP) or 100 μM mersalyl, respectively.

Reagent	Concn. (mM)	Asp/Glu carrier		ADP/ATP carrier	
		rel. antiport activity (%)	rel. efflux activity (%)	rel. antiport activity (%)	rel. efflux activity (%)
PLP	20	62	61	n.d.[b]	n.d.
PLP[a]	20	4	7	1	5
EDC	3	10	34	48	93
SITS	0.5	32	87	n.d.	n.d.
DIDS	0.08	5	108	n.d.	n.d.
DEPC	0.08	5	23	88	87

[a] Activity assay without previous removal of reagent.
[b] Not determined.

completely blocked the action of PLP (2 or 4 mM, respectively). This competitive behavior provides evidence for an interaction of the inhibitior at the substrate binding site. The K_i for PLP was about 0.4 mM.

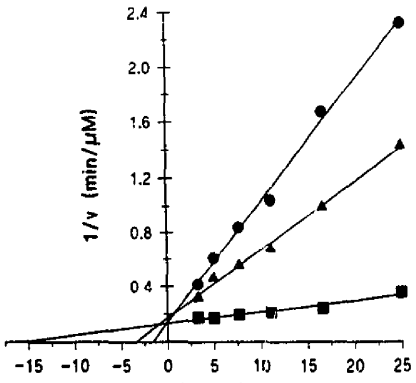
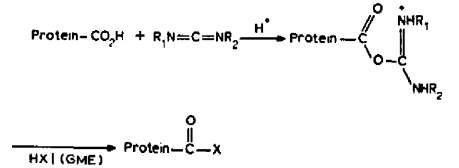


Fig. 1. Competitive interaction of PLP and aspartate with the reconstituted Asp/Glu carrier. The activity of aspartate/aspartate exchange (16 mM internal/40–300 μM external) was determined in the absence (■) and in the presence of PLP (▲, 2 mM or ●, 4 mM) by the forward exchange method. The results are shown in a Lineweaver-Burk plot. Apparent K_m values for aspartate were obtained as follows: 63 (■), 293 (▲) or 624 μM (●). From these data a K_i for PLP of 0.44 mM was calculated.

This is consistent with the apparent IC_{50} (concentration leading to half-maximum inhibition) of 0.9 mM determined in the presence of 50 μM aspartate, i.e. at half saturation (not shown). A significant reaction of PLP with the α-amino group of aspartate at pH 6.5 can be ruled out due to the high pK (9.8) of this group. Considering, moreover, the excess of PLP this putative reaction cannot be responsible for the protective effect observed.

Carbodiimide-catalyzed acylation

When proteoliposomes in Mops/acetate buffer (pH 6.5 or below) were incubated with the hydrophilic carbodiimide 1-ethyl-3-(3-dimethylaminopropyl)carbodiimide (EDC) (10 mM) for 20 min, a complete inhibition of the Asp/Glu carrier was observed. This inhibition, like that caused by PLP, also applied to the uniport (efflux) activity of the carrier (see Introduction), albeit with less efficiency (Table I). The inactivation by EDC was essentially dependent on the presence of acetate in the incubation medium (Fig. 2). At least equimolar acetate concentrations were needed to obtain maximum inhibition. This observation is in clear contrast to the classical reaction of carbodiimides described in the literature [27], i.e. carbodiimides reacting with intrinsic COOH-groups of the protein (Eqn. 1). According to this reaction mechanism added (extrinsic) carboxylates should counteract the effect of EDC, since they compete with the intrinsic carboxyls.



(1)

Therefore the inactivation of the Asp/Glu carrier by EDC had to occur according to a different mechanism of action. We supposed an *O*-acetylisourea derivative as a reactive intermediate (Eqn. 2).

Glycine methyl ester (GME) in the classical reaction is used as an extrinsic nucleophile (HX in Eqn. 1) in order to be coupled to the EDC-activated carboxyl of the protein, thereby stably modifying aspartate or glutamate side-chains under amide formation. The inactivation of the Asp/Glu carrier, however, was not facilitated by GME but, on the contrary, was prevented. This can be recognized in Fig. 4A from the fact that at simultaneous addition of EDC and GME (0 min incubation) the transport activity was not inhibited. GME obviously led to a reaction of the acetylisourea derivative without participation of the protein. From these findings the chemical mechanism of a carbodiimide-activated acylation was derived, which leads to the

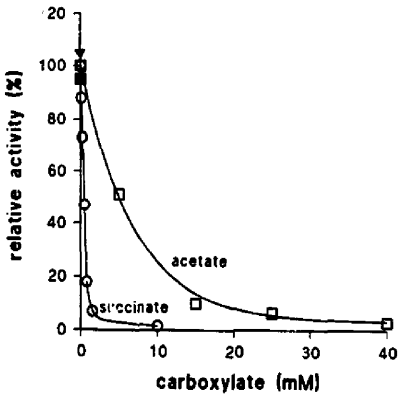
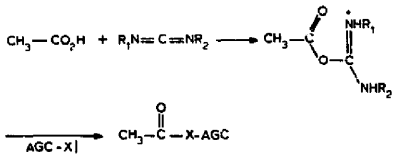


Fig. 2. Dependence of carrier inactivation by EDC on carbonic acids. Proteoliposomes were incubated for 6 min at different concentrations of acetate (□, 0–40 mM) or succinate (○, 0–10 mM) with 20 or 10 mM EDC, respectively. After removal of non-reacted reagents by gel filtration the residual exchange activity of the Asp/Glu carrier was measured. In the absence of acetate or succinate EDC showed no effect, i.e. the transport activities determined differed not significantly from the controls measured without EDC (■, 40 mM acetate; ▼, 0 mM acetate). Since the data were obtained from two separate experiments (with acetate or succinate), they were expressed as percentage of the activity measured in the absence of the respective carbonic acid.

modification of nucleophilic groups (X|) of the Asp/Glu carrier protein (Eqn. 2).



(2)

TABLE II

Influence of carboxylates and nucleophiles on the inactivation of the reconstituted Asp / Glu carrier by EDC

The inhibition of antiport activity (forward exchange) was determined after incubating proteoliposomes with EDC at pH 6.2 in the presence of carbonic acids and amino acids or their derivatives as indicated. Experiment A, incubation with 5 mM EDC for 15 min; experiment B, incubation with 2.5 mM EDC for 6 min.

	Carboxylate	p*K*	Nucleophile	rel. antiport activity (%)
A	–		–	100
	10 mM ammonium acetate	4.8	–	30
	10 mM sodium acetate	4.8	–	33
	10 mM propionate	4.9	–	33
	10 mM oxoglutarate	≤ 5 [a]	–	35
	10 mM succinate	5.7 [a]	–	0
	10 mM citrate	6.4 [a]	–	1
	10 mM ammonium acetate	4.8	5 mM lysine	100
B	15 mM succinate		–	23
	15 mM succinate		5 mM lysine	33
	15 mM succinate		5 mM lysine-OMe [b]	77
	15 mM succinate		5 mM glysine-OMe [c]	91
	15 mM succinate		5 mM *N*-acetyl-cysteine	52

[a] Last ionisation step.
[b] Lysine methyl ester.
[c] Glycine methyl ester.

Testing the dependence of inactivation on the kind of the carboxyl compound, it turned out that dicarboxylates or tricarboxylates like succinate or citrate were much more efficient than acetate or propionate (Table

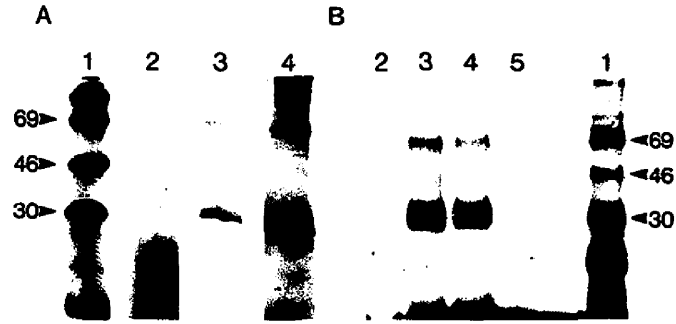


Fig. 3. ^{14}C-Succinylation of lysine residues of the Asp/Glu and the ADP/ATP carrier. The EDC-catalyzed incorporation of [^{14}C]succinate into the Asp/Glu (68 kDa) and ADP/ATP carrier protein (30 kDa) is shown, as was observed after incubating proteoliposomes (internal 16 mM aspartate/10 mM ATP) at pH 6.2 with 10 mM EDC/0.25 mM [^{14}C]succinate (A) or with 12 mM EDC/0.12 mM [^{14}C]succinate (B). In A the time of incubation was varied stopping the reaction by 40 mM GME: 0 min (lane 2, simultaneous addition of EDC and GME), 8 min (lane 3) and 15 min (lane 4). In B the time of labelling was 10 min. Previously, however, the proteoliposomes were treated with 10 mM PLP (lane 2), water (lane 3), 25 μM diethyl pyrocarbonate (lane 4) or 1 mM SITS (lane 5) for 10 min at pH 6.2. A, B: After removal of unreacted reagents by gel filtration the proteins were extracted from the liposomes (see Methods, samples of B without precipitation) and about 3 μg were separated electrophoretically on a SDS-gel. The labelled bands were visualized fluorographically on X-ray films (see Methods). In lane 1 ^{14}C-labelled molecular weight marker proteins were applied (molecular mass in kDa).

II and Fig. 2). This finding can be explained by the higher pK values of succinic (pK_2 5.7) and citric acid (pK_3 6.4) as compared to acetic acid (pK 4.8) leading to a more effective activation of the respective groups by EDC at pH 6.2. Consistently, a hydrogen bonding of the protonated carboxyl to the carbodiimide system was put forward as a first step in the addition reaction [28]. Using succinic acid an IC_{50} of 0.5 mM was determined (Fig. 2).

As a proof for the suggested mechanism Fig. 3A shows the EDC-catalyzed incorporation of [^{14}C]succinate into the carrier protein. Proteoliposomes were incubated with 10 mM EDC/0.25 mM [^{14}C]succinate for 7.5 and 15 min, i.e. under conditions leading to specific inhibition of the Asp/Glu carrier (IC_{50} 0.5 mM) but not of the coreconstituted ADP/ATP carrier (IC_{50} 5 mM, data not shown). Nevertheless, both proteins (the 68 and 30 kDa band, respectively) were labelled. In either case the ^{14}C-succinylation was completely suppressed by the presence of extrinsic nucleophiles like GME.

GME could thus be used as a reagent stopping the modification reaction. This facilitated to investigate the kinetics of carrier inactivation in more detail. At non-limiting concentration of the carboxyl compound (50 mM acetate) k_{app}, the apparent first-order inactivation constant (with respect to the protein), is a function of the inhibitor concentration. Eqn.3 is valid for low EDC concentrations:

$$k_{app} = c[\mathrm{EDC}]^n$$

or

$$\log k_{app} = \log c + n \log[\mathrm{EDC}] \quad (3)$$

where n is the mean reaction order with respect to EDC and c is a proportionality factor (cf. Ref. 29). The k_{app} values could be derived from the slope of the inactivation kinetics that were plotted semi-logarithmically in Fig. 4A. The data obtained showed a linear dependence of k_{app} on the EDC concentration at least up to 1 mM (not shown). Thus, the rate-limiting inactivation step followed a first-order reaction also with respect to EDC ($n \approx 1$). By a double-logarithmic secondary plot (cf. Eqn. 3) n could be determined (Fig. 4B) [29,30]. We obtained a value of about 0.7 for the inactivation of antiport and likewise for the inactivation of efflux, which is consistent with the modification of a single amino acid residue leading to inactivation of the functional carrier molecule. In total a second-order reaction was monitored with inactivation constants k_2 of 260 $min^{-1}M^{-1}$ (antiport) and 40 $min^{-1}M^{-1}$ (efflux), respectively.

The most important question concerned the nature of the functional group modified (X in Eqn. 2). This group in the protein environment must show a relatively low pK in order to react, at pH 6.2, in the deprotonated form as a nucleophile, as is illustrated in Table II B. Various nucleophiles, when added to the incubation mixture, showed different effectiveness in competing with the intrinsic nucleophile to be modified. Adding 5 mM lysine (pK^ϵ 10.5–10.9) only pro-

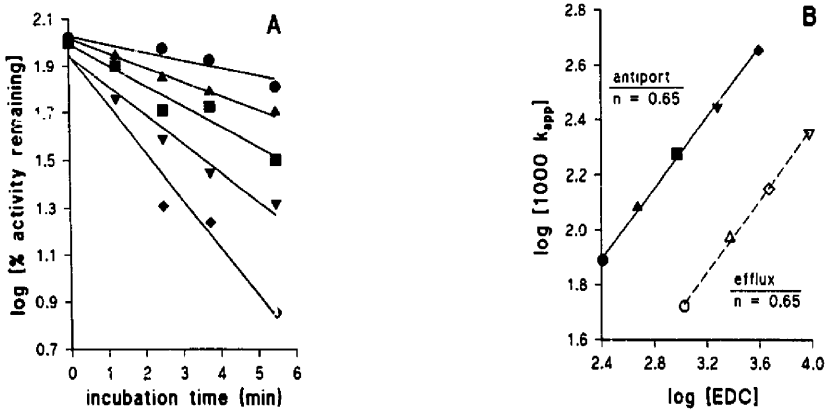


Fig. 4. Kinetics of inactivation of the reconstituted Asp/Glu carrier by EDC/acetate. Proteoliposomes, prelabelled with [^{14}C]aspartate (see Methods), were incubated at pH 6.2 with different EDC concentrations (●, 0.25 mM; ▲, 0.5 mM; ■, 1 mM; ▼, 2 mM; ◆, 4 mM) for different times as indicated. The acetylation reaction (50 mM acetate) was initiated by addition of EDC and stopped by 20 mM GME. To control samples both compounds were added simultaneously ($t = 0$). After gel filtration the remaining antiport activity was measured (addition of 1.5 mM aspartate, backward exchange). The rates are given as percentage of controls (A). In a double-logarithmic secondary plot (B) of the apparent inactivation constants k_{app} (2.303·slope of the straight lines of A) vs. the applied EDC concentration a linear relationship was obtained corresponding to Eqn. 4. From the slope of this straight line the mean reaction order n of the rate-limiting inactivation step (with respect to EDC) could be derived (closed symbols). In addition, the results of inactivation of the efflux activity are shown (open symbols).

tected from inactivation when less effective carboxyls were used in moderate excess to EDC. On the contrary, lysine methyl ester led to a protection under almost all conditions. Hence the free COOH-group of lysine was activated by EDC, which could result either in a lysylation of the protein or in the synthesis of polylysine (without participation of the protein). The protective effects of GME and also of *N*-acetylcysteine (Table II B) reflects the lower pK values of the α-amino group (pK 7.8 for glycine ethyl ester) and the SH-group (pK 8.4 for cysteine). Thus, the results shown are in agreement with those of model reactions revealing that a modification of SH- and even of amino groups is absolutely feasible.

Since the Asp/Glu carrier has highly reactive cysteines [15,16], we had to test as to whether SH-reagents could protect from modification by EDC/succinate. However, pretreatment with $HgCl_2$, *p*-(chloromercuri)-benzoate or 5,5'-dithiobis(2-nitrobenzoate) did not substantially change the observed inhibition by EDC, as was measured after finally removing bound SH-reagent by dithioerythritol (data not shown).

The question of lysine modification was addressed by investigating the mutual interaction of the reversibly binding PLP (see above) and EDC with the Asp/Glu carrier. In functional experiments it turned out that after pretreatment with 10 mM PLP an inhibition by 10 mM EDC/2.5 mM succinate was hardly detectable (Table III). Likewise 4-acetamido-4'-isothiocyanostilbene-2,2'-disulfonate (SITS), another lysine-reagent (see below), competed with PLP (Table III) and, as will be shown, with EDC/succinate. The mutual displacement of PLP and EDC at the same reactive lysine was proven by labelling experiments (Fig. 3B). Pretreating the proteoliposomes with 10 mM PLP completely suppressed the labelling by 12 mM EDC/0.12 mM [^{14}C]succinate both in the Asp/Glu (68 kDa) and in the ADP/ATP carrier protein (30 kDa). A pretreatment with 1 mM SITS likewise showed an effective

TABLE III

Effect of EDC / succinate, SITS, DIDS and DEPC on the reconstituted Asp / Glu carrier after modification by PLP

Proteoliposomes first were pretreated for 2 min with 10 mM PLP (or with incubation buffer) at pH 6.5 and subsequently incubated for 3–5 min (SITS 15 min) with the indicated reagents. After gel filtration the residual antiport activity was determined by forward exchange measurements. The results obtained from multiple experiments (mean values ± S.D.) are given in percentage of the PLP-treated or of the untreated control, respectively. The inhibition caused by PLP was reverted by 40% after gel filtration (cf. Table I).

Addition (before gel filtration)	Antiport activity after gel filtration (% of control)	
	PLP present	PLP absent
10 mM EDC/ 2.5 mM succinate	83 ± 16	14 ± 9 (n = 3)
160 μM SITS	104	54
680 μM SITS	90	23
10 μM DIDS	67 ± 13	57 ± 13 (n = 3)
50 μM DEPC	96 ± 6	16 ± 11 (n = 5)

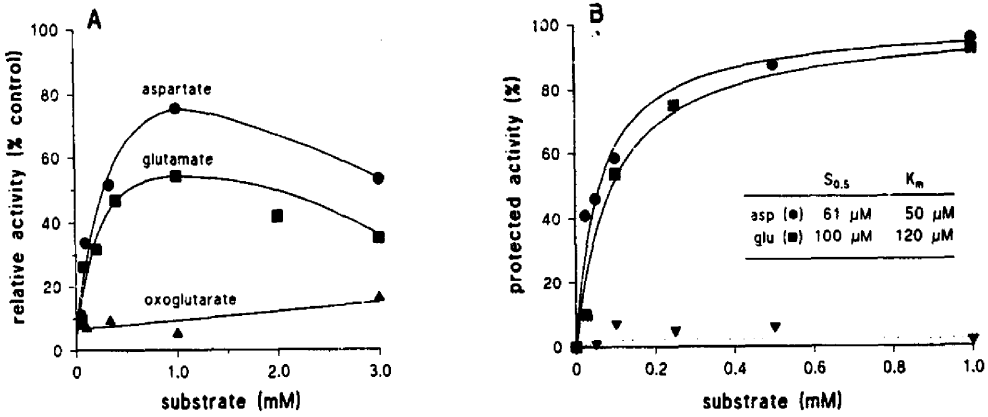


Fig. 5. Substrate protection against inactivation by EDC. (A) Proteoliposomes were incubated for 4 min at pH 6.2 with 6 mM EDC/50 mM acetate in the presence of aspartate (●), glutamate (■) or oxoglutarate (▲) (0–3 mM). After gel filtration the residual antiport activity was measured by the forward exchange method (50 μM external [^{14}C]aspartate). The calculated rates are given as percentage of the untreated control. In B succinate (5 mM) was used as the carboxyl component instead of acetate and the inhibition caused by 2 mM EDC (4 min incubation) in the presence of aspartate (●), glutamate (■) or DL-α-aminoadipinate (▼) (0–1 mM) was examined. The residual antiport activities this time are expressed as protected activities, i.e. in percentage of the total inhibitable activity measured without substrate protection (maximum inhibition: 28% residual activity = 0% protected activity). The dependence of this protected activity on the concentration of the protector in the case of aspartate and glutamate could be fitted by the following equation: protected activity (%) = 100 [protector]/($S_{0.5}$ + [protector]). The calculated $S_{0.5}$ values are given in the inset and are compared to the respective K_m values (see text).

reduction of [^{14}C]succinate incorporation, which, however, led to some residual (and selective) labelling of the Asp/Glu carrier band. Further inhibitors of the Asp/Glu carrier like diethyl pyrocarbonate (see below), not at all prevented the reaction of EDC/succinate with the protein (Fig. 3B).

Thus, the lysine residue at the binding center should be the crucial one for inactivation by EDC, as was observed in the case of PLP. Consistently, the inhibition by EDC was considerably reduced, if aspartate or glutamate ($\leq$ 1 mM) was present during incubation (Fig. 5). This protective effect was substrate-specific. Neither α-aminoadipinate, the next homologue of glutamate, showed a comparable effect nor did asparagine, α-aminobutyrate, α-oxoglutarate or other similar chemicals. In order to achieve an almost complete substrate protection the more effective carboxyl component succinate had to be used (Fig. 5B); when using acetate (Fig. 5A) residual inhibition was observed that obviously was due to activation by EDC of the β-(γ-)carboxyl of aspartate (glutamate). With succinate a 50% protection was obtained at a substrate concentration ($S_{0.5}$) of 61 μM aspartate or 100 μM glutamate, respectively. These values correspond exactly to the transport affinities (K_m values) of the carrier for the respective substrates, i.e. 50 μM (aspartate) and 120 μM (glutamate) at pH 6.2 [7].

Modification by isothiocyanostilbenedisulfonates

In order to substantiate the existence of a functional lysine at the active site of the Asp/Glu carrier we selected isothiocyanates as lysine-specific probes. These reactive compounds lead to products that are stable against hydrolyzation only when reacting with amino groups to form thiourea derivatives [32,33]. For several anion carriers in particular the isothiocyanostilbenedisulfonates 4-acetamide-4'-isothiocyanostilbene-2,2'-disulfonate (SITS) and 4,4'-diisothiocyanostilbene-2,2'-disulfonate (DIDS) have proven as effective and specific modifiers [34–37] (see also references in Ref. 38), the former having one, the latter having two reactive NCS-groups.

Also in the case of the Asp/Glu carrier DIDS turned out to be a potent, rapidly reacting inhibitor (IC_{50} 10 μM, pH 6.5). At neutral pH DIDS was by far more effective than phenylisothiocyanate (IC_{50} 3 mM), fluorescein 5-isothiocyanate (IC_{50} 450 μM) and even than SITS (IC_{50} 300 μM), the monofunctional analogue of DIDS (Table I). Likewise 4,4'-dinitrostilbene-2,2'-disulfonate (DNDS), though carrying no isothiocyanate group, showed a clear inhibitory effect on the reconstituted transport activity (IC_{50} 200 μM) which, however, turned out to be solely caused by competition of DNDS with the substrate. This suggests that stilbenedisulfonates have a certain affinity for the substrate binding site. The reversible interaction of DNDS, however, did not protect from the irreversible inactivation caused by DIDS or EDC/succinate (not shown).

In order to characterize the lysine(s) modified by the isothiocyanates the competition with PLP and EDC was investigated in detail. For a clear interpretation of these experiments it was favorable to know whether also in the case of SITS and DIDS only one lysine had to be modified to inhibit the carrier. Therefore we

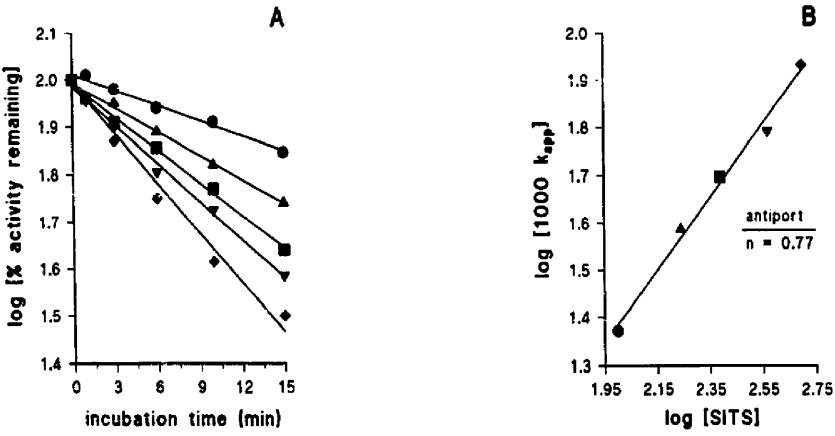


Fig. 6. Kinetics of inactivation of the reconstituted Asp/Glu carrier by SITS. Proteoliposomes (internal 16 mM aspartate), were incubated at pH 6.8 with different SITS concentrations (●, 0.1 mM; ▲, 0.175 mM; ■, 0.25 mM; ▼, 0.375 mM; ♦, 0.5 mM) for different times as indicated. The modification reaction was initiated by addition of SITS and stopped by 5 mM DTE. To control samples both compounds were added simultaneously (t = 0). After gel filtration the remaining antiport activity was measured by the forward exchange method adding 50 μM [^{14}C]aspartate. The rates are given as percentage of controls (A). In a double-logarithmic secondary plot (B) of the apparent inactivation constants k_{app} vs. the applied SITS concentration the mean reaction order n (with respect to SITS) was derived obtaining a value of 0.77; for explanation see legend of Fig. 4.

studied the inactivation kinetics in experiments similar to those shown in Fig. 4 for EDC. As a reagent capable to block the action of isothiocyanates dithioerythritol (DTE) proved to be well-suited (cf. Ref. 39). Applying this stopping reagent first-order inactivation kinetics (with respect to the protein) could be demonstrated in the case of SITS (Fig. 6A), whereas inactivation by DIDS showed two kinetic components (not shown). In Fig. 6B the mean reaction order n with respect to SITS was determined yielding a value of about 0.8. Thus the modification of one lysine residue, like in the case of EDC, is sufficient to inactivate the carrier protein. The second-order rate constant k_2 for inactivation by SITS was 91 $min^{-1} M^{-1}$.

On the basis of this information the finding that pretreatment of the carrier protein by SITS protects from EDC-catalyzed ^{14}C-succinylation (Fig. 3B) means that SITS (like PLP) and EDC modify the same lysine residue. These structural data were corroborated by functional data (Table III) demonstrating that, after modification by PLP, SITS does not inactivate the Asp/Glu carrier. On the contrary, in the case of DIDS a reduction of its effectiveness by PLP could not at all be noticed (Table III), although DIDS (100 μM) like SITS suppressed the protein labelling by EDC/[^{14}C]succinate (not shown). These findings argue for a different reaction mechanism of DIDS.

The difference between the interaction of SITS and DIDS with the protein also was reflected by substrate protection experiments. Adding aspartate or glutamate to the incubation medium led to no protection from inactivation by DIDS (not shown), whereas complete protection was observed in the case of SITS, as can be recognized from Fig. 7. Other compounds chemically related to aspartate or glutamate showed no influence on the extent of modification (not shown). From Fig. 7 $S_{0.5}$ values were derived, namely 90 μM for aspartate and 260 μM for glutamate, respectively, reflecting the transport affinities (K_m values) of the carrier for the two different substrates (see Fig. 7). In this respect it has to be noted that the carrier is characterized by a higher apparent K_m for glutamate at pH 6.5 (Fig. 7) than at pH 6.2 (Fig. 5) [7].

Modification by diethyl pyrocarbonate (DEPC)

DEPC ethoxyformylates the imidazole nitrogen atoms of histidines with remarkable selectivity [19,40]. In proteins side-reactions with lysines and, in some cases, with tyrosines were found when using higher DEPC concentrations (usually $\geq$ 1 mM).

Asp/Glu antiport was effectively inhibited by DEPC at pH values between 6.2 and 7.0. DEPC proved to be the most selective reagent with respect to its action on the Asp/Glu carrier (IC_{50} 20 μM, pH 6.5) and on the coreconstituted ADP/ATP carrier ($IC_{50} \geq 200$ μM, pH 6.5). This differential effectiveness applied also to

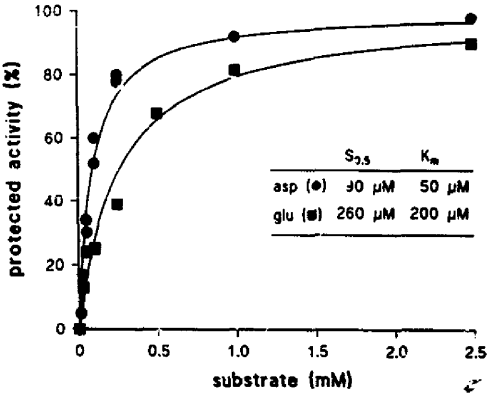


Fig. 7. Substrate protection against inactivation by SITS. Proteoliposomes were incubated for 5 min at pH 6.5 with 300 μM SITS in the presence of different concentrations of aspartate (●) or glutamate (■). After gel filtration the residual antiport activity was measured by the forward exchange method (50 μM external [^{14}C]aspartate). The calculated rates are given as protected activities, i.e. in percentage of the total inhibitable activity measured without substrate protection (maximum inhibition: 48% residual activity = 0% protected activity). For determination of $S_{0.5}$ values see legend of Fig. 5B.

the efflux activities of the two carriers, although the inhibition of aspartate efflux even at higher DEPC concentrations was less complete than the inhibition of aspartate antiport (Table I).

Upon additon of histidine to the incubation medium the inactivation by DEPC was stopped only at $\geq$ 200-fold excess of histidine over reagent. Thus, under kinetic control the reaction of DEPC with the protein involved functional groups of higher reactivity than that of free histidine. By using histidine in millimolar concentrations as a blocking reagent the kinetics of transport inactivation caused by 10–75 μM DEPC could be resolved in the time range of 0.5 to 8 min. In the same way as elucidated in Figs. 4 and 6 the mean reaction order n with respect to DEPC was determined (data not shown). The value of 0.8 obtained is consistent with a reaction of one DEPC per functional carrier molecule that is sufficient for transport inactivation. For the total reaction a second-order inactivation constant k_2 of about 6800 $min^{-1} M^{-1}$ was determined.

After modification by 20 μM DEPC the inhibited carrier could not be reactivated by treatment with 100 mM hydroxylamine (45 min, pH 7) (not shown). This result, on the one hand, elucidates that no essential tyrosine residue was modified [19]. Since, however, on the other hand also (mono-) ethoxyformylated histidines should be attacked by NH_2OH [41], this finding can be taken as evidence that not histidine but lysine residues were modified by DEPC. Support to this

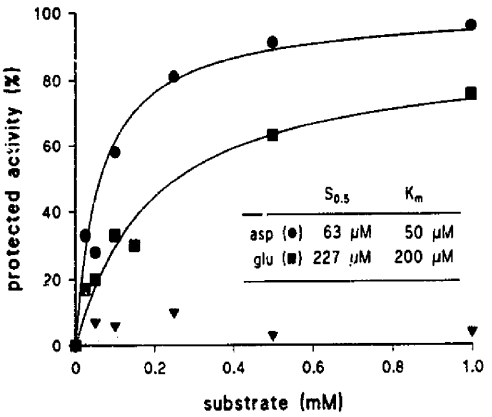


Fig. 8. Substrate protection against inactivation by DEPC. Proteoliposomes were incubated for 8 min at pH 6.5 with 20 μM DEPC in the presence of 0–1 mM aspartate (●), glutamate (■) or DL-α-aminoadipinate (▼). After gel filtration the residual antiport activity was measured by the forward exchange method (50 μM external [^{14}C]aspartate). The calculated rates are given as protected activities, as explained in the legend of Fig. 5B (maximum inhibition: 37% residual activity = 0% protected activity). For determination of $S_{0.5}$ values see also Fig. 5B.

suggestion came from functional studies investigating the effect of carrier pretreatment by the lysine reagent PLP. As shown in Table III, PLP caused a pronounced protection of the carrier from inhibition by DEPC. An effect of PLP to this extent likewise was observed in the case of EDC and SITS. On the contrary, Fig. 3B demonstrates that when reagents were added in the opposite order, i.e. DEPC (25 μM) first, there was no interference at all with the subsequent labelling of lysines by EDC/[^{14}C]succinate. Experiments to resolve the question of the DEPC target using labelled DEPC were not successful due to the low specific radioactivity of [^{14}C]DEPC that is commercially available.

One observation made when using high concentrations of [^{14}C]DEPC (1 mM), nevertheless, was that 10 mM aspartate (but not 10 mM ATP) protected from labelling mainly of the 68 kDa band assigned to the Asp/Glu carrier (not shown). Thus, another point in common with PLP, EDC and SITS obviously was the reaction of DEPC at the external binding center of the reconstituted carrier protein. This conclusion more directly had to be drawn from substrate protection experiments (Fig. 8). In fact, specificity and concentration dependence of the protection observed corresponded to that of the transport ($S_{0.5}$(aspartate) = 63 μM, $S_{0.5}$(glutamate) = 227 μM).

Discussion

The negatively charged substrates of the Asp/Glu carrier suggest basic groups at the two binding centers of the translocator. Considering membrane proteins positively charged side-chains often are found at the interface of transmembrane helices and connecting loops [42], as was proposed also for the ADP/ATP carrier [43]. This could be of importance for the transport event assuming that these charges line the entrance of the postulated transmembrane channel of the carrier thereby increasing its affinity for the substrate. In the case of the ADP/ATP carrier at least three lysine side-chains were connected with substrate binding and translocation, since their chemical reactivity was correlated to the state of transport function [44]. The binding sites, however, could not definitely be localized in the amino acid sequence of any mitochondrial carrier. In this respect first results were obtained by photoaffinity labelling experiments [45,46].

As shown in the present paper lysine- and histidine-modifying reagents caused complete inhibition of the Asp/Glu carrier (Table I). Substrate protection experiments (Figs. 5, 7 and 8) led to the conclusion that the affected residues are located at the exofacial binding site. Further support to these results came from the mutual competition of the different binding site inhibitors applied, as was reflected by experiments based on transport function (Table III) as well as by labelling experiments (Fig. 3B).

An alternate mechanism of chemical modification by carbodiimides

Carbodiimides are well-known modifiers of carboxylic side-chains which, after activating the COOH-group at slightly acidic pH, normally lead to amide formation with available amino group-nucleophiles [27]. Actually, the same basic reaction was observed in the experiments described here, as becomes apparent when comparing Eqns. 1 and 2. The difference, however, was that an (intrinsic) nucleophile was modified instead of the carboxyl by coupling to this group an (extrinsic) carboxyl instead of the nucleophile. The result is an acylation of an amino group (Eqn. 2), namely that of a lysine side-chain, as had to be concluded from the coincident data provided by functional (Table III) and labelling experiments (Fig. 3B). The unusual with this reaction occurring at a pH of 6.2 certainly is the extraordinary high reactivity of the affected ε-amino group which, though present in a very low concentration, is not completely prevented from modification when adding extrinsic nucleophiles in large excess (Table IIB). This necessitates a pK unexpectedly low for a lysine. In the microenvironment of a protein, however, pK values and chemical reactivities can change considerably [31]. In the literature only recently a citrylation of a lysine residue of the anion exchanger from erythrocytes was described that was catalyzed (at pH 6.8) by the very similar carbodiimide 1-ethyl-3-(3-trimethylaminopropyl)carbodiimide (ETC) [47]. The reaction mechanism of ETC was formulated according to

Eqn. 2. The affected lysine could be addressed to be lys *a*, which was known to be selectively modified by DIDS at neutral pH (p*K* 7–8 [48]).

Similar to these published results the Asp/Glu and the ADP/ATP carrier were protected from ^{14}C-succinylation mediated by EDC, if the proteins were preincubated with other lysine-reagents, namely PLP, SITS or DIDS. A selective labelling of the 68 kDa band assigned to the Asp/Glu carrier could be achieved when choosing an appropriate SITS concentration that predominantly suppressed the subsequent labelling by EDC/[^{14}C]succinate of the ADP/ATP carrier band (Fig. 3B). The specific protection from inactivation by the substrate (Fig. 5), however, did not lead to a selective suppression of the labelling of any band (not shown). Consequently, additional succinylation sites must exist, the modification of which does not cause inhibition of the Asp/Glu carrier. It should be noted that for inactivation only one residue of the functional carrier molecule had to be modified by EDC, as shown by inactivation kinetics (Fig. 4).

One or two positive charges at the exofacial binding center?

Our studies on modification by DEPC (Fig. 8, Table III) demonstrate that also this reagent modifies a functional group at the exofacial binding site. According to the remarkable list of reports on the specificity of DEPC [19,40,41,49–52] the affected group should be a histidine, in particular when taking into account the pH of 6.5 in these experiments and the very low IC_{50} observed. This would lead to the conclusion that a histidine contributes a second positive charge at the active site. Further support to this model is provided by the observation that 4-bromophenacyl bromide, another histidine-modifying reagent [53,54], inactivates the Asp/Glu carrier very efficiently ($IC_{50} \approx 3$ μM, data not shown).

On the contrary, there is provoking evidence in favor of the highly reactive lysine to be modified not only by PLP, EDC and SITS but also by DEPC, thus leading to a binding site model with only one reactive basic group. First, the observed non-reversibility of DEPC-mediated inactivation by hydroxylamine and, second, the protection from inactivation by pretreatment of the carrier with PLP (Table III) are strong arguments for lysine modification. Nevertheless, there are alternative explanations. The missing reactivation by NH_2OH, on the one hand, could be the result of irreversible damage of the histidine side-chain. NH_2OH can open the imidazole ring when previously ethoxyformylated at both nitrogen atoms [19,55]. The protective effect of PLP, on the other hand, could be either due to reaction of DEPC with the free OH-group of PLP or due to sterical hinderance at juxtaposed lysine and histidine residues. Vice versa, DEPC did not restrict the labelling of lysine by EDC/[^{14}C]succinate (Fig. 3B). But also this result is no unequivocal proof against a reaction with a lysine, since in a labelling experiment the modification by EDC of additional, not essential and not DEPC-reactive groups may be registered.

For a final decision whether the lysine or a histidine was modified by DEPC more conclusive competition experiments would be necessary using labelled DEPC. As explained above, our experiments carried out with [^{14}C]DEPC, due to low specific radioactivity, were not suitable for clear interpretation. If this histidine exists at the binding site it would be very interesting to find out as to whether this group is involved in the regulation of the carrier's substrate affinity by pH. For the H^+-binding site of the Asp/Glu carrier, prerequisite for glutamate/H^+ cotransport, we previously estimated from kinetic analyses a p*K* of 6.5, which might reflect the participation of a histidine side-chain [7,8]. However, in our investigations of the DEPC-modified carrier so far no indication could be obtained of a kinetic parameter that specifically was altered concerning the transport of glutamate and not of aspartate (data not shown).

Further topographical relationships

Most of the binding site modifiers applied (except SITS and DIDS) inhibited not only the antiport but also the uniport (efflux) activity of the Asp/Glu carrier (Table I). Obviously, after modification of the lysine and the putative histidine the inducibility of efflux by SH-reagents [15] was restricted. This suggests that at least one of the two functional cysteines characterized previously [15,16] is structurally related to the basic residue(s) at the active site. Actually, it was found in these earlier studies that the second SH-group relevant for efflux induction was exposed only in the absence of external substrate [16]. This SH-group clearly is not directly modified by the applied lysine and histidine reagents, since inactivation by EDC, SITS, DIDS or DEPC could not be suppressed by more than 35% upon carrier pretreatment with several SH-reagents (see Results). Vice versa, when treating the protein first with EDC, PLP or DEPC, strong (but again not complete!) effects on efflux activity, i.e. cysteine modification, were observed (Table I). These obviously sterical effects can be explained assuming a structural vicinity of the respective groups.

The data shown in the present paper emphasize that the profound change in the transport mechanism caused by efflux induction is due to an interference with the so-called 'intrinsic binding' of the substrate during translocation, as was discussed previously [16]. This intrinsic binding, which is tightly connected with antiport coupling, obviously is correlated with the correct position of cysteines at or close to the exofacial

binding center characterized by a lysine and possibly by a histidine residue of functional importance. In order to find a topographic model describing the sterical relationships of the mentioned functional groups, that specifically can be addressed by the different reagents, more information is needed concerning the two cysteines. Further structural insights into this important domain of the carrier may be gained when succeeding to explain the rather different effects of the two analogue reagents SITS and DIDS, which are likely to be due to the second NCS-group of DIDS. These questions are currently under investigation.

Acknowledgements

The authors appreciate the continuous support of Prof. H. Sahm (Jülich). This work was supported by grants from the Deutsche Forschungsgemeinschaft and the Fonds der Chemischen Industrie.

References

1 LaNoue, K.F. and Schoolwerth, A.C. (1984) in Bioenergetics (Ernster, L., ed.), pp. 221–263, Elsevier, Amsterdam.
2 Meijer, A.J. and Van Dam, K. (1981) in Membrane Transport (Bonting, S.L. and De Pont, J.J.H.H.M., eds.), pp. 235–255, Elsevier, Amsterdam.
3 Krämer, R. and Palmieri, F. (1989) Biochim. Biophys. Acta 974, 1–23.
4 Krämer, R., Kürzinger, G. and Heberger, C. (1986) Arch. Biochem. Biophys. 251, 166–174.
5 Dierks, T. and Krämer, R. (1988) Biochim. Biophys. Acta 937, 112–126.
6 Sluse, F.E., Evens, A., Dierks, T., Duyckaerts, C., Sluse-Goffart, C.M. and Krämer, R. (1991) Biochim. Biophys. Acta, 1058, 329–338.
7 Dierks, T., Riemer, E. and Krämer, R. (1988) Biochim. Biophys. Acta 943, 231–244.
8 Dierks, T. and Krämer, R. (1989) in Anion Carriers of Mitochondrial Membranes (Azzi, A., Nalecz, K.A., Nalecz, M.J. and Wojtczak, L., eds.), pp. 99–110, Springer Verlag, Berlin.
9 Sluse, F.E., Ranson, M. and Liébecq, C. (1972) Eur. J. Biochem. 25, 207–217.
10 Indiveri, C., Dierks, T., Krämer, R. and Palmieri, F. (1991) Eur. J. Biochem. 198, 339–347.
11 Sluse, F.E., Sluse-Goffart, C.M. and Duyckaerts, C. (1989) in Anion Carriers of Mitochondrial Membranes (Azzi, A., Nalecz, K.A., Nalecz, M.J. and Wojtczak, L., eds.), pp. 183–195, Springer-Verlag, Berlin.
12 Aquila, H., Link, T.A. and Klingenberg, M. (1987) FEBS Lett. 212, 1–9.
13 Runswick, M.J., Powell, S.J., Nyren, P. and Walker, J.E. (1987) EMBO J. 6, 1367–1373.
14 Runswick, M.J., Walker, J.E., Bisaccia, F., Iacobazzi, V. and Palmieri, F. (1990) Biochemistry 29, 11033–11040.
15 Dierks, T., Salentin, A., Heberger, C. and Krämer, R. (1990) Biochim. Biophys. Acta 1028, 268–280.
16 Dierks, T., Salentin, A. and Krämer, R. (1990) Biochim. Biophys. Acta 1028, 281–288.
17 Krämer, R. and Heberger, C. (1986) Biochim. Biophys. Acta 863, 289–296.
18 Schägger, H. and Von Jagow, G. (1987) Anal. Biochem. 166, 368–379.
19 Miles, W.E. (1977) Methods Enzymol. 47, 431–442.
20 Krämer, R. (1984) FEBS Lett. 176, 351–354.
21 Churchich, J.E. (1965) Biochemistry 4, 1405–1410.
22 Rippa, M., Spanio, L. and Pontremoli, S. (1967) Arch. Biochem. Biophys. 118, 48–57.
23 Hartman, F.C. and Norton, I.L. (1977) Methods Enzymol. 47, 479–498.
24 Anderson, B.M., Anderson, C.D. and Churchich, J.E. (1966) Biochemistry 5, 2893–2900.
25 Piszkiewicz, D. and Smith, E.L. (1971) Biochemistry 10, 4538–4544.
26 Cabantchik, I.Z., Balshin, M., Breuer, W. and Rothstein, A. (1975) J. Biol. Chem. 250, 5130–5136.
27 Carraway, K.L. and Koshland, D.E., Jr. (1972) Methods Enzymol. 25, 616–623.
28 Blake, P. (1980) in The Chemistry of Ketenes, Allenes and Related Compounds (Patai, S., ed.), pp. 309–362, Interscience/J. Wiley, New York.
29 Levy, H.M., Leber, P.D. and Ryan, E.M. (1963) J. Biol. Chem. 238, 3654–3659.
30 Scrutton, M.C. and Utter, M.F. (1965) J. Biol. Chem. 240, 3714–3723.
31 Cohen, L.A. (1970) in The Enzymes, Vol. 2 (Boyer, P.D., ed.), pp. 147–211, Academic Press, New York.
32 Maddy, A.H. (1964) Biochim. Biophys. Acta 88, 390–399.
33 Hartman, F.C. and Wold, F. (1966) J. Am. Chem. Soc. 88, 3890–3891.
34 Cabantchik, I.Z. and Rothstein, A. (1972) J. Membr. Biol. 10, 311–330.
35 Jentsch, T.J., Keller, S.K., Koch, M. and Wiederholt, M. (1984) J. Membr. Biol. 81, 189–204.
36 Rumpho, M.E., Edwards, G.E., Yousif, A.E. and Keegstra, K. (1988) Plant Physiol. 86, 1193–1198.
37 Gross, A., Brückner, G., Heldt, H.W. and Flügge, U.I. (1990) Planta 180, 262–271.
38 Passow, H. (1986) Rev. Physiol. Biochem. Pharmacol. 103, 61–223.
39 Genchi, G., Patrone, G., De Palma, A., Cambria, A. and Palmieri, F. (1988) Biochim. Biophys. Acta 936, 413–420.
40 Ovádi, J., Libor, S. and Elödi, P. (1967) Acta Biochim. Biophys. Hung. 2, 455–458.
41 Melchior, W.B., Jr. and Fahrney, D. (1970) Biochemistry 9, 251–258.
42 Dalbey, R.E. (1990) Trends Biochem. Sci. 15, 253–257.
43 Klingenberg, M. (1989) Arch. Biochem. Biophys. 270, 1–14.
44 Bogner, W., Aquila, H. and Klingenberg, M. (1986) Eur. J. Biochem. 161, 611–620.
45 Boulay, F., Lauquin, G.J.M., Tsugita, A. and Vignais, P. V. (1983) Biochemistry 22, 477–484.
46 Mayinger, P., Winkler, E. and Klingenberg, M. (1989) FEBS Lett. 244, 421–426.
47 Werner, P.K. and Reithmeier, A.F. (1988) Biochim. Biophys. Acta 942, 19–32.
48 Kampmann, L., Lepke, S., Fasold, H., Fritzsch, G. and Passow, H. (1982) J. Membr. Biol. 70, 199–216.
49 Osterman-Golkar, S., Ehrenberg, L. and Solymosy, F. (1974) Acta Chem. Scand. B28, 215.
50 Garrison, C.K. and Himes, R.H. (1975) Biochem. Biophys. Res. Commun. 67, 1251.
51 Burstein, Y., Walsh, K.A. and Neurath, H. (1974) Biochemistry 13, 205.
52 Bagert, U. and Röhm, K.H. (1989) Biochim. Biophys. Acta 999, 36–41.
53 Crestfield, A.M., Stein, W.H. and Moore, S. (1963) J. Biol. Chem. 238, 2413–2420.
54 Volwerk, J.J., Pieterson, W.A. and De Haas, G.H. (1974) Biochemistry 13, 1446–1454.
55 Avaeva, S.M. and Krasnova, V.I. (1975) Bioorg. Khim. 1, 1600.