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Kinetic discrimination of two substrate binding sites of the reconstituted dicarboxylate carrier from rat liver mitochondria

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The kinetic interaction of various substrates and inhibitors with the dicarboxylate carrier from rat liver mitochondria was investigated using the isolated and reconstituted carrier protein. Due to their inhibitory interrelation the ligands could be divided into two classes: dicarboxylates, sulphate, sulphite and butylmalonate on the one hand and phosphate, thiosulphate and arsenate on the other. The mutual inhibition of substrates or inhibitors taken from one single class was found to be competitive, whereas the kinetic interaction of ligands when taken from the two different classes could be described as purely non-competitive. The half-saturation transport constants K_m and the corresponding inhibition constants K_i of one single ligand, either used as substrate or as inhibitor, respectively, were found to be very similar. These kinetic data strongly support the presence of two different binding sites at the dicarboxylate carrier for the two different classes of substrates considering the external side of the reconstituted protein. When these two sites were saturated simultaneously with malate and phosphate, the turnover of the carrier was considerably reduced, hence indicating that a non-catalytic ternary complex is formed by the two substrates and the carrier molecule.

Introduction

Experimental data obtained in intact mitochondria already 17 years ago suggested the presence of two separate substrate binding sites at the cytosolic side of the dicarboxylate carrier, one specific for phosphate and the other specific for dicarboxylates [1]. Later, other anions were also found to be transport substrates of this mitochondrial carrier system, namely sulphate, sulphite and thiosulphate [2,3], and evidence was obtained that sulphate interacts with the dicarboxylate binding site of the carrier [4]. Now, the purified carrier protein is available in functional active state [5], which offers the possibility to elucidate further these interesting properties of the dicarboxylate carrier in the reconstituted system, thereby characterizing the specificity and number of binding sites.

In the accompanying paper [6] the basic kinetic data of the dicarboxylate carrier, purified from rat liver mitochondria and reconstituted into liposomes, have been described. The present paper evaluates the functional properties of the reconstituted carrier with respect to three different kinds of substrate, i.e., dicarboxylates, phosphate or arsenate and sulphur-containing anions. The mutual influence of these ligands on the transport activity of the carrier was tested at the external side of the proteoliposomes.

Materials and Methods

Materials. Hydroxyapatite (Bio-Gel HTP) and Dowex AG1-X8 were purchased from Bio-Rad, Amberlite XAD-2 from Fluka, L-[U-^{14}C]malate and [^{32}P]phosphate from Amersham International, U.K., egg-yolk phospholipids (L-α-phosphatidylcholine from fresh turkey egg yolk) from Sigma. Other reagents were obtained as reported [1,5]. All other chemicals were of the highest purity commercially available.

Isolation of the dicarboxylate carrier. The dicarboxylate carrier was purified as described previously (see accompanying paper, Ref. 6).

Reconstitution of the dicarboxylate carrier. The procedures for the reconstitution of the dicarboxylate carrier were described in detail in the accompanying paper [6].

Transport measurements. The transport measurements were carried out as described in the accompany-

Abbreviations: Pipes, 1,4-piperazinediethanesulphonic acid; SDS, sodium dodecyl sulphate.

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ing paper [6]. All the curves shown in the figures were drawn by means of a computer-fitting program based on linear regression analysis.

Other methods. Protein was determined by the Lowry method modified for the presence of Triton [7].

Results

Analysis of transport inhibition by substrate analogues

In the first step of kinetic analysis, we chose the two inhibitors arsenate and butylmalonate as representative analogues for the substrates phosphate and dicarboxylate, respectively. The inhibition by these analogues of the exchange of malate (external)/phosphate (internal) and of phosphate/phosphate was studied using different external substrate and inhibitor concentrations. As shown in Fig. 1A, butylmalonate inhibited the uptake of malate in the reconstituted system in a purely competitive manner. In this case we observed a K_i of 0.4 mM for butylmalonate. It has to be pointed out that all other dicarboxylates tested, including malonate, succinate, phthalate, benzylmalonate and phenylsuccinate, were identified as competitive inhibitors of the malate/phosphate exchange by proving their exclusive effect on K_m without changing V of the malate uptake. By using Dixon plots the inhibition constants K_i for these ligands were determined to be 0.44 mM (malonate), 0.82 mM (succinate), 0.95 mM (phthalate), 0.43 mM (benzylmalonate) and 1.6 mM (phenylsuccinate) (experiments not shown).

When, however, the uptake of phosphate was inhibited by butylmalonate (Fig. 1B), the type of inhibition was clearly non-competitive, leading to a K_i of 0.4 mM for butylmalonate. Again, a similar non-competitive pattern of inhibition was found using malonate or succinate as inhibitors for the phosphate/phosphate exchange (experiments not shown). Exactly the opposite situation was observed when the inhibition by arsenate was analyzed. Arsenate inhibited the uptake of phosphate competitively (Fig. 1D) and showed a purely non-competitive type of inhibition when uptake of malate was measured (Fig. 1C). In either case of non-competitive interaction with the carrier (Fig. 1B and C), the common intersection point was located at the abscissa, indicating that the inhibitor binds both to the free carrier and to the substrate-loaded carrier protein with the same half-saturation constant.

Mutual inhibition between malate and phosphate

For more direct characterization of the transport kinetics of the dicarboxylate carrier, the two ligands, so far used as single transport substrates, were added simultaneously thereby acting as mutual inhibitors. It has been shown above that dicarboxylates inhibit malate uptake competitively. In contrast to this, addition of phosphate led to a purely non-competitive type of inhibition (Fig. 2A). When, vice versa, phosphate/phosphate exchange was recorded and malate was added to recontituted liposomes (Fig. 2B), the kinetic analysis revealed a non-competitive relationship of these two

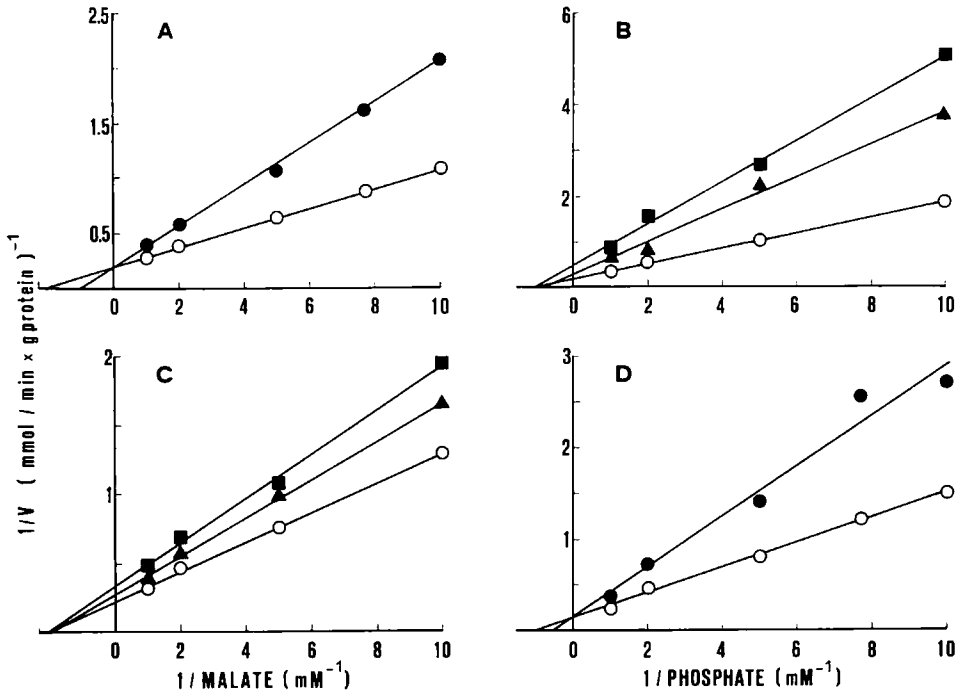

Fig. 1. Inhibition of malate and phosphate uptake in phosphate-loaded liposomes by butylmalonate (A and B) and by arsenate (C and D). Additions: (○) none; (●) 0.4 mM, (▲) 0.3 mM or (■) 0.6 mM butylmalonate; (▲) 0.4 mM, (■) 0.8 mM or (●) 0.6 mM arsenate.

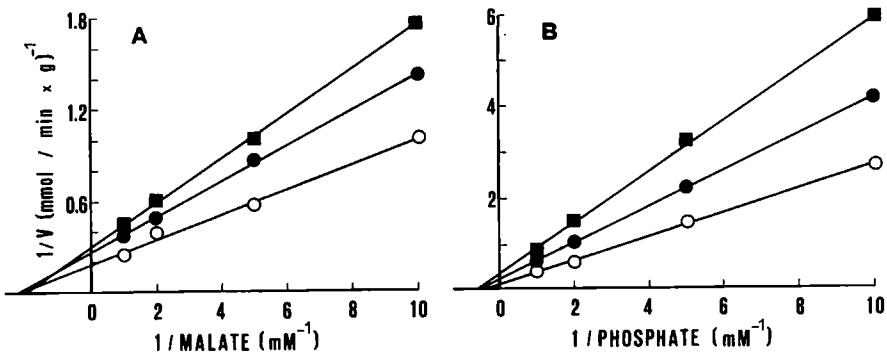

Fig. 2. Inhibition of (A) malate uptake in phosphate-loaded liposomes by phosphate and (B) of phosphate uptake by malate. Additions: (○), none; (●) 0.3 mM or (■) 0.6 mM phosphate; (●) 0.2 mM or (■) 0.4 mM malate.

ligands. The K_i values of the inhibitors phosphate and malate, respectively, could be determined by plotting the data of Fig. 2 according to Dixon [8] (figures not shown). Thereby the inhibition constant K_i of phosphate with respect to malate uptake was found to be 0.8 mM, which is similar to its K_m value as a transport substrate (about 1.4 mM, see also Table II of the accompanying paper [6]). The same held true for malate when used as an inhibitor of phosphate uptake. In this case a K_i value of 0.5 mM was found, which again is very similar to the related half-saturation transport constant K_m of 0.49 mM.

Inhibition of malate and phosphate uptake by sulphur-containing anions

The relatively broad substrate specificity of the dicarboxylate carrier offers the possibility to test also 'non-physiological' substrates of the carrier protein. From experiments in mitochondria it was concluded that also the sulphur-containing anions sulphate, sulphite and thiosulphate are substrates of the dicarboxylate carrier [2,3]. When investigated in the reconstituted system, all three anions inhibited the uptake of malate; differences, however, could be observed with respect to the type of interaction with the carrier. Sulphate and sulphite inhibited competitively, whereas the inhibition by thiosulphate was non-competitive (Fig. 3). The K_i values for the different ligands were calculated to be 0.6, 0.2 and 0.8 mM for sulphate, sulphite and thiosulphate, respectively.

As expected, the opposite effects could be observed when these three compounds were tested for inhibition of phosphate uptake by the reconstituted dicarboxylate carrier (Fig. 4). Thiosulphate was found to be a competitive inhibitor, whereas sulphate and sulphite acted non-competitively. In this case, the K_i values for sulphate, sulphite and thiosulphate were 0.5, 0.3 and 0.4 mM, respectively.

Analysis of the mechanism of transport inhibition

In order to gain insight into the mutual interaction between substrate and inhibitor at the carrier protein it is necessary to distinguish between complete and partial types of inhibition. For this purpose we varied the inhibitor concentration at a fixed substrate concentration. The experimental results were then analyzed according to Webb [9] using double-reciprocal plots of

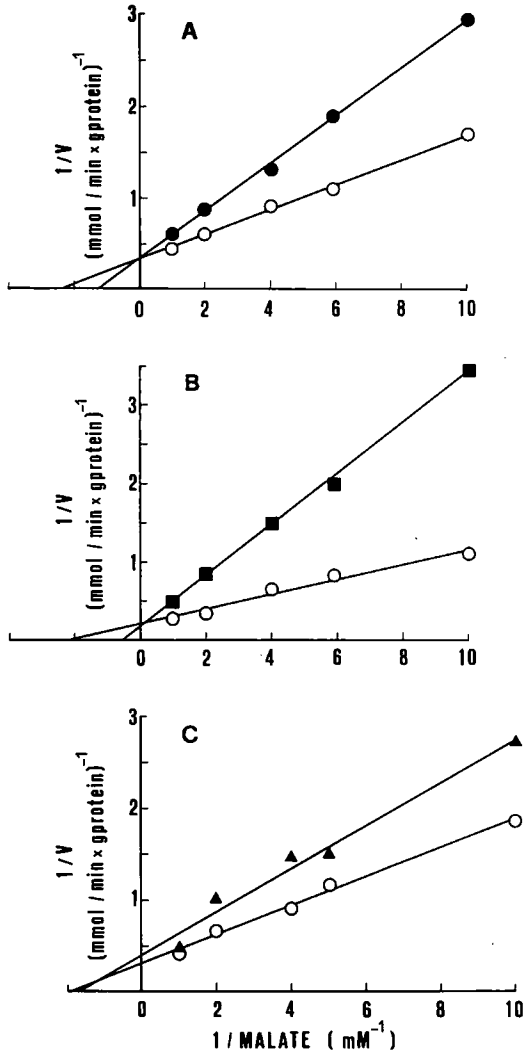

Fig. 3. Inhibition of malate uptake in phosphate-loaded liposomes by sulphate (A), sulphite (B) and thiosulphate (C). Additions: (○) none; (●) 0.6 mM sulphate; (■) 0.6 mM sulphite; (▲) 0.6 mM thiosulphate.

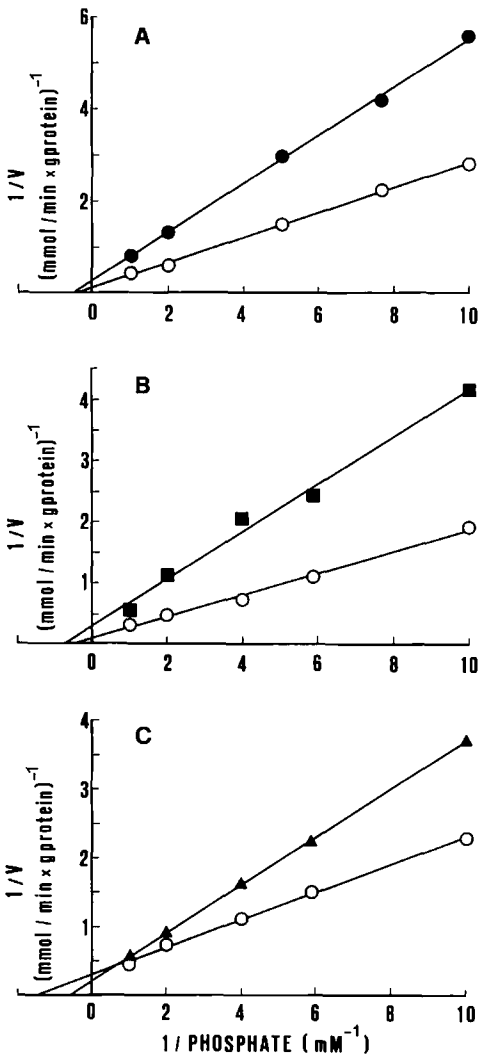

Fig. 4. Inhibition of phosphate uptake by sulphate (A), sulphite (B) and thiosulphate (C). Additions: (○) none; (●) 0.6 mM sulphate; (■) 0.6 mM sulphite; (▲) 0.6 mM thiosulphate.

the fractional inhibition (i) against the concentration of the inhibitor (Fig. 5). The fractional inhibition is defined as $i = 1 - (V_i/V)$, where V_i and V are the rates of uptake in the presence or absence of inhibitor, respectively. Completely competitive or non-competitive types of inhibition lead to straight lines which intersect at the ordinate at unity, whereas partial types of inhibition lead to intercepts of higher values [9].

In the case of both substrates, malate as well as phosphate, all the lines obtained for the different ligands used as inhibitors intersected at the ordinate close to unity as shown in Fig. 5 for sulphate, thiosulphate, malonate and phosphate. These results give clear evidence that the tested ligands are completely competitive and non-competitive inhibitors, respectively, of malate or phosphate uptake catalyzed by the reconstituted dicarboxylate carrier.

The kinetic analysis of the non-competitive inhibition mechanism could further be elaborated by investigating whether the inhibitory effect is caused by the simultaneous binding of the two substrates, malate and phosphate, to the two different sites of the same carrier molecule, thus forming a non-catalytic complex. If this complex occurs, no translocation of either substrate should take place if saturating conditions of malate and phosphate are provided. Therefore, the total transport activity, i.e., the sum of [^{32}P]phosphate plus [^{14}C]malate transport, was determined when the two substrates were present in different ratios and at different degrees of saturation (0.4 mM and 4 mM) outside the proteoliposomes. The results presented in Fig. 6 demonstrate that the total turnover of the carrier was not at all constant at different ratios of these two ligands, although the total concentration of phosphate plus malate was not changed. Interestingly, the minimum activity was observed at a malate/phosphate ratio of about 1. Moreover, the inhibitory effect was much more pronounced if the respective binding sites were saturated to a higher degree (4 mM, Fig. 6B). The extent of inhibition that was achieved adding 2 mM phosphate/2 mM malate was 40–60% (five experiments).

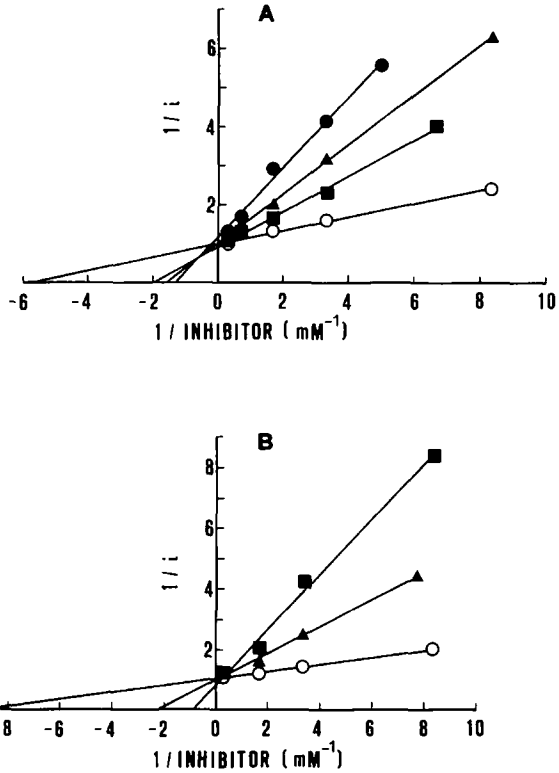

Fig. 5. Inhibition of the uptake of malate (A) and phosphate (B) in phosphate-loaded liposomes by various concentrations of sulphate, thiosulphate, malonate and phosphate. The rates of uptake of 0.1 mM malate (A) and 0.1 mM phosphate (B) were measured. All inhibitors were added simultaneously with the labelled substrate. The symbol i refers to the fractional inhibition (see text). Added inhibitors: (○) malonate; (■) thiosulphate; (▲) sulphate; (●) phosphate.

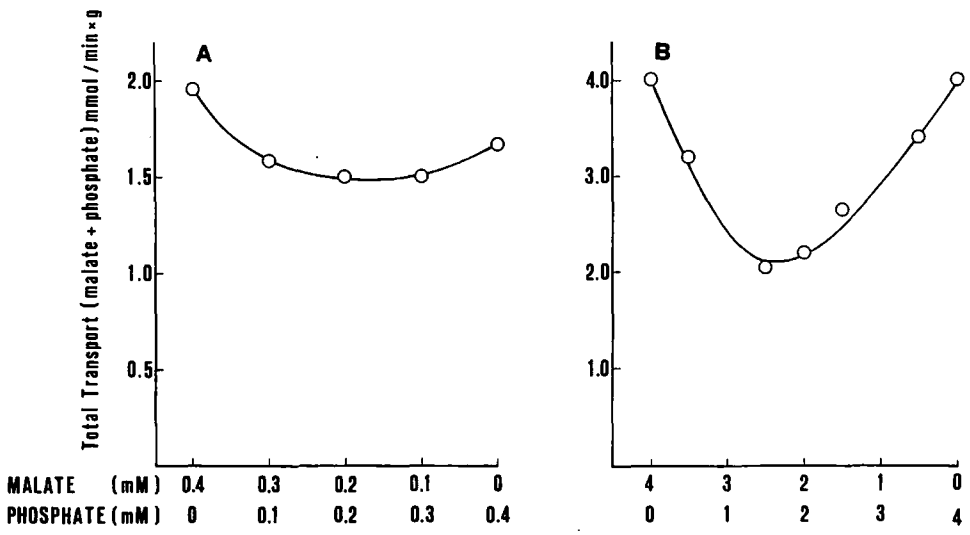

Fig. 6. Inhibition of carrier turnover by simultaneous binding of phosphate and malate to the dicarboxylate carrier. [^{14}C]Malate and [^{32}P]phosphate were simultaneously added to phosphate-loaded liposomes at the concentrations indicated. The total concentration was 0.4 mM (A) or 4 mM (B).

Discussion

For the kinetic analysis of a carrier-mediated transport process involving separate substrate binding sites it is essential to evaluate first the kinetic data of the transport reaction when only one single substrate is present in either compartment. This has been extensively described in the accompanying paper [6]. The most important result in this respect was the finding that the V values of phosphate uptake by the dicarboxylate carrier using phosphate-loaded liposomes (phosphate/phosphate homoexchange) and the corresponding uptake of malate (malate/phosphate heteroexchange) were essentially the same. This intrinsic functional symmetry of the carrier-mediated transport is a prerequisite for analyzing the competitive or non-competitive behaviour of the two substrates when present on the same side of the membrane.

On the basis of experiments with intact mitochondria it has been suggested long ago that the dicarboxylate carrier has two different binding sites, one specific for phosphate and the other for dicarboxylates [1]. The kinetic data obtained in the reconstituted system as reported here support this proposal. The main evidence for this interpretation is the non-competitive nature of the inhibition of phosphate uptake by malate or butylmalonate on the one hand, and the non-competitive inhibition of malate uptake by phosphate or arsenate on the other hand.

Based on this finding we could divide these and various other substrates and inhibitors of the dicarboxylate carrier into two different classes of ligands by kinetic analysis of their inhibitory interrelation. Ligands of the same class showed competitive interaction with the carrier when simultaneously present in the external compartment, whereas ligands of the two different classes clearly interacted in a non-competitive way of mutual inhibition. Non-competitive inhibition is generally accepted to be due to, at least partially, different binding sites. Thus, the ligands malate, succinate, phenylsuccinate, malonate, butylmalonate, benzylmalonate, phthalate, sulphate and sulphite on the one hand, and phosphate, arsenate and thiosulphate on the other, were correlated with the two different binding sites at the external surface of the dicarboxylate carrier. It has to be taken into account that a more detailed characterization of the translocation mechanism depends on the analysis of the orientation of the reconstituted dicarboxylate carrier protein within the liposomal membrane. This question is currently under investigation. However, since all substrates are known to be transported in the two possible directions, which means that both binding sites must be present at both sides of the membrane, even a mixed orientation of the reconstituted carrier protein would not significantly influence the basic conclusions as drawn in this paper. The observations of non-competitive inhibition mechanisms cannot result from different binding sites located on differently oriented carrier proteins, which consequently means that the two binding sites reside on the same carrier molecule. Nevertheless it should be stated that the two different sites, the interaction of which is investigated in this paper, have to be accessible from the outside of the proteoliposomes.

When the data concerning the observed cases of non-competitive inhibition were analyzed using reciprocal plots, i.e. Lineweaver-Burk as well as Dixon plots, the point of intersection was always located on the abscissa. This formally means that $K_i = K_{ii}$, which can be interpreted by assuming that the binding (not the

transport!) of the substrate to the carrier is not influenced by the presence of the respective inhibitor, and vice versa. Furthermore, the nature of the non-competitive inhibition, as described here for the reconstituted dicarboxylate carrier, most easily is interpreted to be due to the presence of a non-catalytic, i.e., not transport-competent, ternary complex that is formed by the simultaneous rapid binding of both substrate and inhibitor to the carrier protein at the external side. This can be rationalized directly from Fig. 6, showing that not only the transport activity concerning one single substrate, but also the total transport activity of the dicarboxylate carrier, i.e., the sum of activity for the transport of phosphate and malate (one so far formally treated as inhibitor), was clearly reduced if the two ligands were present at about the same concentration. Provided that the two different binding sites of the carrier are saturated with substrate, this ternary complex can be assumed to be completely inactive, as became evident when the concentration of substrates was increased from 0.4 to 4 mM (Fig. 6). Due to methodical reasons this kind of experiment could not be carried out at concentrations at which both binding sites of the dicarboxylate carrier were fully saturated. Therefore, the maximum inhibition was not achieved. When similar studies were undertaken investigating the effect of ATP plus ADP on the translocation rate of the reconstituted ADP/ATP carrier, no significant decrease in turnover number was found (Krämer, R., unpublished results). These results obtained with the reconstituted dicarboxylate carrier are consistent with a non-competitive inhibition mechanism that includes the formation of a non-translocating ternary carrier-malate-phosphate complex.

It should be noted that this interpretation of the mechanism of substrate and/or inhibitor interaction with the dicarboxylate carrier seems to be different from the situation observed in the case of other mitochondrial carrier proteins. The kinetic analysis of both the oxoglutarate carrier [10] and the aspartate/glutamate carrier [11] revealed a common binding site for all the substrates transported specifically by these carrier proteins.

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