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INHIBITION OF THE MITOCHONDRIAL PHOSPHATE CARRIER BY A REACTION WITH A CARBOXYL GROUP REAGENT

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The effect of N-ethyl-5-phenylisoxazolium 3"-sulfonate (Woodward's reagent K, WRK), a reagent forming covalent bonds with protein carboxyl groups, on the activity of the mitochondrial phosphate carrier was investigated. Treatment with WRK of mitochondria or of extracted carrier incorporated into liposomes, inhibited phosphate transport in a reconstituted liposomal system. Increasing the binding of WRK resulted in increased inhibition: the modified carrier protein showed a reduced affinity for phosphate, but binding of WRK had no effect on the V_{max} of phosphate transport. It was concluded that WRK caused a conformational change in the carrier protein not involving the phosphate or H^+ carrier sites such that its affinity for phosphate was lowered.

A carrier protein exists in the inner mitochondrial membrane for the transport of phosphate into mitochondria: this phosphate carrier has been purified (1,2) and its amino acid sequence has been determined (3,4).

Phosphate is transported together with a stoichiometric amount of H^+ (or in exchange with OH^-). One could visualize a negatively charged site (such as glutamic or aspartic acid) functioning simultaneously as a necessary (but not sufficient) binding-site for H^+ in the transport of phosphate. If that were the case, it should be possible to inhibit phosphate transport by blocking glutamic or aspartic acids on the carrier protein by covalent binding of an inhibitor. Such a carboxyl-binding reagent has recently been described (5).

This report deals with the inhibition of phosphate transport, using the carrier protein in a reconstituted liposomal system, with the inhibitor N-ethyl-5-phenyllisoxazolium 3'-sulfonate (Woodward's reagent K, WRK). This reagent binds covalently to carboxyl groups, which can then be reduced to alcohol groups with labelled sodium borohydride, thus labeling the carrier protein (5).

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MATERIALS AND METHODS

Materials. Woodward's Reagent K was obtained from Sigma Corp. Hydroxyapatite (Bio-Gel HTP) and Dowex AG1-X8 were purchased from Bio-Rad, egg yolk phospholipids from Fluka and cardiolipin from Avanti-Polar Lipids. [^{32}P]phosphate and [^{3}H]sodium borohydride (7.2 Ci/mmol) were obtained from the Radiochemical Center (Amersham, U.K.). All other reagents were of the highest purity commercially available.

Incubation with WRK and isolation of phosphate transport protein. Frozen beef heart mitochondria, prepared as described in ref. 6, were thawed and washed once in 20 mM KCl/20 mM KH_2PO_4/1 mM EDTA (pH 6.5). The pellet (about 12.5 mg mitochondrial protein) was suspended in the same buffer and WRK solution (10 mg per ml in the same ice-cold buffer) was added to make a final volume of 1 ml with the desired concentration of WRK. The suspension was then left at room temperature for the desired time, usually 10 min. After washing twice with the above buffer, the mitochondrial pellet was suspended in 600 µl of the above buffer containing 2.5% Triton X-114. After 20 min at 0°C the suspension was centrifuged at 147000 x g for 15 min to obtain a clear supernatant referred to as extract. From this point on, the purification of the extract, preparation of liposomes, incorporation of the phosphate transport protein into liposomes and assay of phosphate transport into proteoliposomes, were carried out exactly as described in a previous report from this laboratory (1). Transport was initiated by adding to the proteoliposomes [^{32}P]PO_4^{-3} (20 mM, approx. 200,000 cpm) and terminated after 1 min by addition of 2mM N-ethylmaleimide (NEM), an inhibitor of the phosphate carrier. In control samples NEM was added 1 min before the labeled phosphate. The activity of phosphate uptake was calculated by subtracting the control from the experimental values. The NEM-insensitive radioactivity associated with the control samples was in all experiments less than 15% with respect to the NEM-sensitive phosphate uptake and was not affected by WRK. Essentially similar results were obtained when freshly-prepared (intact) mitochondria were used and the incubation with WRK was carried out in an isotonic medium (sucrose, 220 mM; KH_2PO_4, 20 mM; Hepes, 20 mM; EGTA, 1 mM; pH, 6.5; in absence of phosphate, sucrose was 250 mM).

RESULTS

When mitochondrial suspensions were treated with WRK and extracted with Triton X-114 buffer, and the extract incorporated into liposomes without further purification, the resulting proteoliposomes, assayed for phosphate transport activity by exchange of [^{32}P]phosphate into the liposomes, showed inhibition of transport. As shown in Fig. 1A, increasing concentration of WRK caused increasing inhibition, with 91% inhibition obtained at 2.2 mM WRK. Essentially the same results were obtained when mitochondria were treated with WRK, extracted and the extract purified over hydroxyapatite (1) (Fig. 1B). When the extract of untreated mitochondria (without purification on hydroxyapatite) was first incorporated into liposomes and the proteoliposomes treated with WRK, similar inhibition of the transport activity was observed (not shown). Inhibition of transport activity by WRK was dependent on time of treatment with WRK. In order to obtain accurate treatment times, the reaction of WRK with the mitochondria was stopped by rapidly making the suspension 10 mM with respect to glutamic acid. The effectiveness of glutamic acid as a stopping reagent was verified as shown in Table I. Maximum inhibition of transport activity was observed 10 min after addition of WRK to the mitochondria (data not shown).

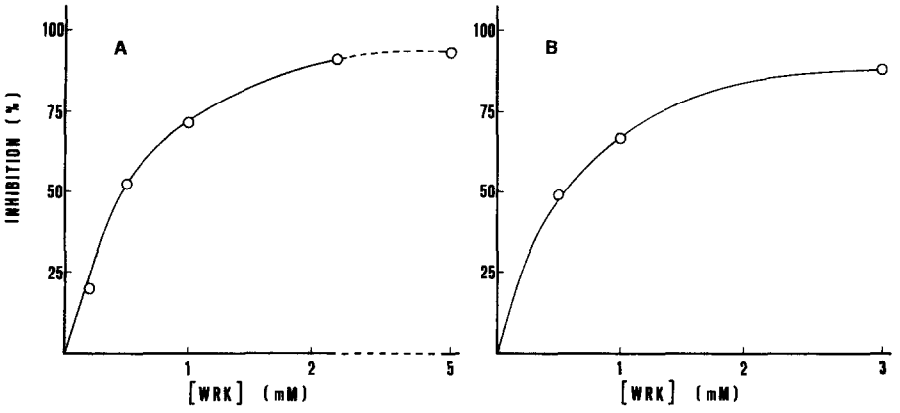

Fig. 1. Inhibition of phosphate transport by treatment of mitochondria with WRK: effect of WRK concentration. Incubation (time, 15 min) carried out as described under Materials and Methods. (A) Mitochondria extracted and the extract incorporated directly into liposomes. Phosphate transport assayed by uptake of labeled phosphate into liposomes, as described in the text. (B) The same experiment, except that the mitochondrial extract was purified over hydroxyapatite before incorporation into liposomes, as described in the text. Specific activity, 8.9 μmoles exchanged/min/mg protein.

To demonstrate that the substrate (phosphate) did not inhibit (competitively or otherwise) the reaction of WRK with the phosphate carrier, nor that WRK reacted preferentially with phosphate, the reaction of mitochondria with WRK was carried out in presence and absence of phosphate, both with respect to WRK concentration and time: Figs. 2A and 2B show that there was no significant difference in the inhibition, whether phosphate was present or not.

The effect of phosphate concentration on the transport of phosphate into unloaded liposomes containing the purified phosphate carrier, modified by reaction with WRK, was

TABLE 1

VERIFICATION THAT GLUTAMIC ACID ACTS AS REAGENT TO STOP THE INHIBITION OF PHOSPHATE TRANSPORT BY WRK

	+ Glutamic acid	- Glutamic Acid
WRK	$[^{32}P]PO_4^{-3}$ into liposomes	$[^{32}P]PO_4^{-3}$ into liposomes
mM	cpm	cpm
0	3034	3484
0.5	3833	1104
1.0	3397	583

Mitochondria were suspended in the buffer described under Materials and Methods and treated for 30 min with different concentrations of WRK as shown, at the same time as with glutamic acid (final concentration, 10 mM) (or without, as shown). Mitochondrial extracts were then incorporated into liposomes and transport was assayed by adding $[^{32}P]PO_4^{-3}$.

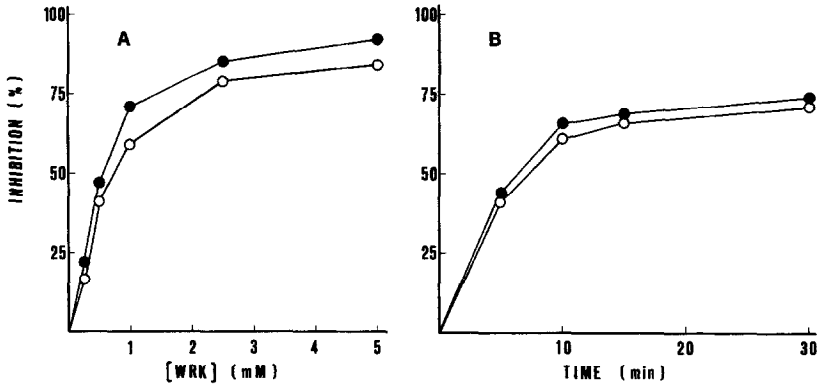

Fig. 2. Inhibition of phosphate transport by treatment of mitochondria with WRK in presence and absence of phosphate: (A) effect of WRK concentration. Treatment with WRK in presence of phosphate was done as in experiment shown in Fig. 1B; treatment without phosphate was done in presence of 20 mM Hepes instead of phosphate. (B) Effect of treatment time. Incubation with WRK in presence of phosphate (full circles) in the following buffer: sucrose (220 mM), KH_2PO_4 (20 mM), Hepes (20mM), EGTA (1 mM), pH 6.5. In absence of phosphate (open circles): sucrose, (250 mM), Hepes (20mM), EGTA (1 mM), pH 6.5. WRK concentration, 0.5 mM. Mitochondrial extracts were purified over hydroxyapatite before incorporation into liposomes, as in Fig. 1B. Specific activity, 27.5 and 28.0 µmoles exchanged/min/mg protein in A and B respectively.

investigated. Fig. 3 shows that increased WRK attachment resulted in increased inhibition of phosphate transport. This increased inhibition by WRK was caused by an apparent reduced affinity of the protein for phosphate. There was no effect on the V_{max} of

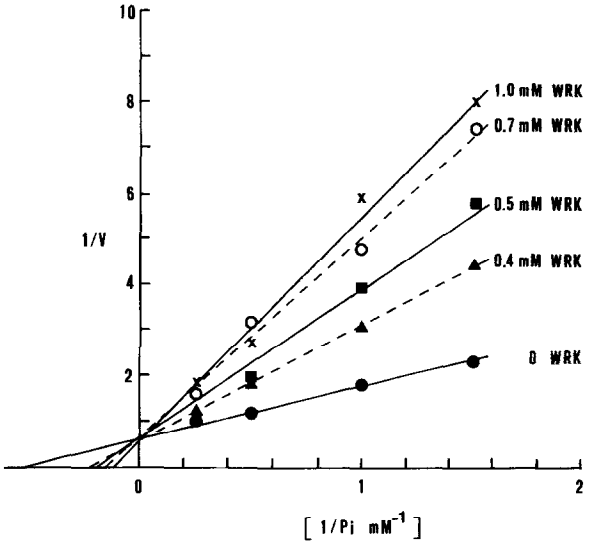

Fig. 3. Inhibition of the phosphate carrier by treatment of mitochondria with WRK; effect of phosphate concentration on transport in the reconstituted system. Incubations (times, 15 min) carried out as described under Materials and Methods. Concentrations of WRK as indicated in the figure. The mitochondrial extract was purified over hydroxyapatite before incorporation into liposomes. Assay of $[^{32}P]PO_4^{-3}$ uptake by unloaded liposomes: Liposomes were prepared by sonication of 100 mg/ml egg yolk phospholipids in a buffer containing 50 mM KCl, 20 mM Hepes and 2 mM EGTA (pH 6.5) (i.e. in the absence of phosphate). Phosphate uptake was initiated by adding to the proteoliposomes $[^{32}P]$-phosphate at the concentrations indicated. V is expressed as µmoles/min/mg protein.

phosphate transport: rate of transport when the protein was saturated by phosphate was maximal whether the protein was modified by WRK or not.

In order to label the inhibitor-protein complex, [^{3}H]$NaBH_4$ was added to the mitochondria which had reacted with WRK. After washing, the phosphate carrier was solubilized and purified, and its radioactivity was determined. It was found that the radioactivity associated with the purified phosphate carrier protein was linearly proportional to the concentration of WRK (data not shown). The curve obtained however, did not correspond to that found when measuring inhibition against concentration of WRK (Fig. 1B), presumably because carboxyl groups in addition to or other than those involved in phosphate transport were reacting with WRK.

DISCUSSION

From these results it is clear that WRK inhibits phosphate transport activity, both with mitochondria and when the phosphate carrier was extracted and reconstituted in liposomes, presumably by covalent reaction of the inhibitor with glutamic and/or aspartic acid (5). Inhibition correlated with concentration of inhibitor, with maximal inhibition attained after 10 min of treatment. Similar results were obtained with freshly-prepared (intact) mitochondria.

The location on the carrier protein of the glutamic/aspartic acid residues which bind WRK covalently, is currently unknown. The simplest assumption would be that they bind protons which are co-transported into mitochondria with phosphate. If that were the case, then the co-transport of H^+ would be a necessary step in the transport of phosphate. However, the results illustrated in Fig. 3 show that, if WRK were attached to the putative H^+ - binding site, one would not expect that high phosphate concentrations could overcome the inhibition by WRK. This inhibitor seems to act like an allosteric modifier: a conformation change seems to take place in a part of the protein not involved in the H^+ or phosphate carrier sites, changing the conformation of the protein such that its affinity for phosphate is lowered, thus modifying the reaction with phosphate.

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