Characterization of a dCTP Transport Activity Reconstituted from Human Mitochondria*

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A protein fraction of mitochondria from human acute lymphocytic leukemia cells, which could be reconstituted into proteoliposomes to have dCTP transport activity, has been partially purified by hydroxyapatite and blue Sepharose chromatography. The dCTP transport activity in proteoliposomes was time-dependent and could be activated by Ca\(^{2+}\) and to a lesser extent by Mg\(^{2+}\). None of the other divalent cations tested could activate the transport activity. Other deoxynucleoside triphosphates could also inhibit the uptake of dCTP with the potency dGTP > dTTP. Although ATP could competitively inhibit dCTP uptake with a \(K_a\) value of 8 \(\mu\)M, the reconstituted dCTP uptake activity was not sensitive to the ATP/ADP carrier inhibitor atracyloside or the sulphydryl reagent N-ethylmaleimide. This suggests that the dCTP transport system studied is not the same as the ATP/ADP carrier. In conclusion, these studies describe the first functionally reconstituted mitochondrial carrier that displays an efficient transport activity for dCTP.

The inner membrane of mitochondria normally possesses very low electrophoretic permeability to most molecules. However, through the action of specific transport systems, the inner mitochondrial membrane is selectively permeable to a number of co-factors, metabolites, and nucleotides (1, 2). The AAC\(^{1}\) is the only mitochondrial nucleotide carrier successfully isolated and reconstituted in an active state (3). The AAC is highly selective for ATP and ADP, mediating the import of ADP from the cytoplasm and export of matrix ATP. The ATP-Mg\(^{2+}\) carrier is another nucleotide transport activity that has been described in isolated mitochondria (4) but has not been successfully isolated. Another member of the family of anion transport proteins that reside within the mitochondrial inner membrane is the phosphate carrier that catalyzes transport of inorganic phosphate into the mitochondrial matrix, where the phosphate is utilized for phosphorylating ADP to ATP (5).

The existence of a mechanism for mitochondrial dNTP uptake has been suggested by DNA synthesis experiments using isolated mitochondria (6–8). These studies indicated that exogenous dNTPs could be utilized by isolated mitochondria to synthesize mitochondrial DNA. In addition, based on previous studies from our laboratory, antiviral nucleoside analog triphosphates in mitochondria appear to originate from the cytoplasm (9). In these studies, the absence of 2',3'-dideoxycytidine-induced mitochondrial toxicity in cytoplasmic dCyd kinase-deficient cells suggested that the cytoplasmic-form metabolite 2',3'-dideoxycytidine triphosphate is the nucleotide source in the inhibition of mitochondrial DNA synthesis. Thus, the 2',3'-dideoxycytidine triphosphate metabolites inside mitochondria appear to originate from the cytoplasm. However, the experimental evidence supporting the existence of a dNTP carrier in intact mitochondria is only circumstantial and not definitive as for other mitochondrial metabolite carriers.

The final proof for the existence of a carrier protein is its isolation and functional reconstitution. To that end, we demonstrate that upon incorporation into lipid vesicles, partially purified mitochondrial protein catalyzes dCTP uptake. This transport activity displays unique substrate specificity and inhibitor sensitivity from that previously described for mitochondrial anion transporters. To our knowledge, this is the first report of a substantially purified preparation of this class of mitochondrial carrier activity in functional form.

EXPERIMENTAL PROCEDURES

Chemicals—Hydroxyapatite (Bio-Gel HPT) was purchased from Bio-Rad. Blue Sepharose and Sephadex G-50 were from Amersham Pharmacia Biotech. Soybean asolectin (1-α-phosphatidylcholine), cholesterol, Triton X-114, Dowex 1 × 4 (100–200 mesh, chloride form), atracyloside, and nucleotides were from Sigma.

Preparation of [\(\gamma\)-\(^{32}\)P]dCTP—Nucleoside diphosphate kinase (from Baker’s yeast; Sigma) was used to prepare [\(\gamma\)-\(^{32}\)P]dCTP from dCDP as follows. A 1-mL mixture containing 10 units of nucleoside diphosphate kinase, 2 mM [\(\alpha\)-\(^{32}\)P]GTP (100 \(\mu\)Ci), and 0.5 mM dCDP in 150 mM Tris acetate, pH 7.5, and 10 mM MgCl\(_2\) was incubated at 37 °C for 10 min. The reaction was stopped by the addition of 3 volumes of ice-cold methanol and incubated on ice for 15 min. After centrifugation at 14,000 \(\times g\) for 10 min, the supernatant containing [\(\gamma\)-\(^{32}\)P]dCTP was evaporated to dryness and resuspended in 500 \(\mu\)L of water. The mixture containing dCDP, GDP, [\(\gamma\)-\(^{32}\)P]GTP, and [\(\gamma\)-\(^{32}\)P]dCTP was chromatographed by ion exchange HPLC using a Whatman Partisol-SAX column (4.6 mm × 25 cm) at a flow rate of 1 mL/min. The nucleotides were resolved with a gradient consisting of water to 30 mM buffer (potassium phosphate, pH 6.7) from 0 to 10 min, 30–150 mM buffer from 10 to 15 min, 150 mM buffer from 15 to 50 min, and 300 mM buffer from 50 to 80 min. Peaks were identified by authentic standards, and the peak corresponding to [\(\gamma\)-\(^{32}\)P]dCTP was collected. The purified [\(\gamma\)-\(^{32}\)P]dCTP was diluted 5-fold with water, placed over a DEAE-Sephadex A-25 column (2-ml bed volume), and washed with 20 mL of water to remove the phosphate buffer and the [\(\gamma\)-\(^{32}\)P]dCTP was eluted with 10 mL of ammonium formate (500 mM). The eluted sample was freeze-dried to remove the ammonium formate and resuspended to a volume of 2 mL in water. The purity of [\(\gamma\)-\(^{32}\)P]dCTP was verified by ion exchange HPLC as described above, and the radiospecificity was determined to be 90 \(\mu\)Ci/mmol.

Preparation of Mitochondria and Partial Purification of Proteins with dCTP Transport Activity—Mitochondria were isolated from acute lymphocytic leukemia cells after leukopheresis of a patient in blast

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1 The abbreviations used are: AAC, ATP/ADP carrier; HPT, hydroxyapatite.
crisis and further purified on a discontinuous sucrose gradient as described previously (9). Frozen mitochondria (100–150 mg) were solubilized for 10 min on ice in 10 ml of a buffer containing 20 mM Hepes (pH 7.0), 1% Triton X-114, 150 mM Na2SO4, 1 mM phenylmethylsulfonyl fluoride, and 1 mM benzamidine. The detergent was separated from the aqueous phase by centrifugation at 4 °C for 1 h at 4 °C. The upper aqueous phase was decanted, and the detergent phase was re-suspended to 10 ml in 20 mM Hepes (pH 7.0), 1 mM phenylmethylsulfonyl fluoride, and 1 mM benzamidine (buffer B). The detergent extract was applied to an HPT column (1 g of dry HPT/20 mg of protein) connected in tandem with a blue Sepharose column (1 ml of Blue Sepharose 2 ml of protein) equilibrated with buffer B. The unbound protein-containing fraction was eluted from the tandem columns with buffer B.

Preparation of Proteoliposomes—Asolectin was further purified as described (10). For use in transport studies, cholesterol (120 mg/ml final concentration) was added to the asolectin (200 mg/ml final concentration). Cholesterol has been reported to prevent protein-mediated leakage in reconstituted proteoliposomes (11). The asolectin was stored in chloroform under nitrogen in a light proof container at −20 °C. Immediately before use, an aliquot of asolectin was dried under a stream of nitrogen. Following the addition of 2 ml of 20 mM Hepes (pH 7.0), the lipid was dispersed by sonication (Branson Sonifier 250; microtip output control = 4; 80% duty cycle; on ice) until the mix appeared transparent.

Aliquots of freshly isolated protein fractions (typically 0.3 mg) were mixed with lipid vesicles (55 mg) to a final volume of 1 ml. This mixture was vortexed and then rapidly frozen in liquid nitrogen. For ATP/ADP exchange reactions and dCTP efflux experiments, proteoliposomes were prepared in the presence of 100 μM ADP and 20 μM [α-32P]dCTP, respectively. These proteoliposomes could be stored at −80 °C up to 5 days without appreciable loss of dCTP transport activity. Immediately prior to assay, the samples were freeze-thawed twice (total of three cycles) using liquid nitrogen and thawed a final time in an ice-water bath followed by sonication (Branson Sonifier 250; microtip output control = 2; 70% duty cycle; 30 burst; total sonication time = 25 s; on ice). For ATP/ADP exchange reactions and dCTP efflux experiments, external radioactivity, respectively, were removed by passing the proteoliposomes over an ion exchange column (Dowex; 1 × 3.5 cm) equilibrated with 20 mM Hepes (pH 7.0) at 4 °C. The first ml of the turbid eluate from the Dowex column was collected and then used for transport experiments. A heterogeneous population of large, primarily unilamellar proteoliposomes suitable for transport studies are generated by this general freeze-thaw-sonicate procedure (12–15).

Adenine nucleotide uptake—All measurements of nucleotide uptake into reconstituted proteoliposomes were carried out at 37 °C. Transport was initiated by the addition of 4 μl of radiolabeled substrate (typically [α-32P]dCTP, 10 μM final concentration, 30 Ci/mmol) to 190 μl of proteoliposomes in a final volume of 200 μl. For competition assays, competitor was added to the desired concentration to the proteoliposome mix before the radiolabeled substrate. After the desired incubation time, the reaction mixture was placed over a Dowex ion exchange column (1 × 3.5 cm) equilibrated with 20 mM Hepes (pH 7.0) at 4 °C in order to remove the external radioactivity not transported into proteoliposomes. The liposomes were eluted with 4 ml of ice-cold equilibration buffer, collected in 16 ml of scintillation fluid, vortexed, and counted. Subtracting values obtained when a 100-fold molar excess of nonradio-labeled substrate was added 2 min prior to the radiolabeled substrate made corrections for nonspecific uptake of radioactivity. For efflux experiments, external radioactivity was removed by passing the samples through a Dowex column and collecting and counting the liposomes as described above. The transport activity was calculated by subtracting the experimental values from the control values (efflux at 4 °C).

RESULTS AND DISCUSSION

Mitochondria possess a variety of specific carrier systems for the transport of metabolites across the inner mitochondrial membrane. Because of the importance of mitochondrial toxicity in nucleoside analog chemotherapy (for a review, see Ref. 16), we have sought to extend our knowledge of dCTP uptake into mitochondria. Our procedure for isolation and reconstitution of active dCTP transport from human mitochondria consisted of three basic steps. First, sucrose gradient isolated mitochondria were extracted with Triton X-114. Second, hydrophobic membrane proteins in the detergent were separated from the aqueous phase. Finally, the detergent phase containing solubilized membrane proteins was chromatographed on HPT and blue Sepharose. When applying these conditions to mitochondria from acute lymphocytic leukemia cells, the activity of dCTP transport was significantly enriched, but it remains a heterogeneous mixture of proteins. In Table I the activity of the protein obtained by blue Sepharose chromatography relative to that of the HPT chromatography is reported. For comparison, we report also the data on the total mitochondrial membrane extract. However, reconstitution of a total membrane extract in liposomes and its comparison with the data obtained from a smaller number of proteins after chromatography should be done with caution and may not be fully reliable because of differences in protein-protein interac-tions, liposome size, and passive permeabilities. Uptake of [α-32P]dCTP could be detected in proteoliposomes reconstituted using crude mitochondrial protein extracts and detergent phase membrane proteins with a specific activity of 2.8 and 18 pmol/min/mg protein, respectively. However, only minimal dCTP transport activity could be detected in proteoliposomes using aqueous phase proteins after detergent phase separation. Approximately 180- and 90-fold increases in the specific activity of dCTP uptake in proteoliposomes compared with crude extracts were obtained after HPT and blue Sepharose chromatography, respectively. The decrease in specific activity after blue Sepharose chromatography compared with HPT eluates may indicate the presence of multiple dCTP transport activities in mitochondrial proteins that are not resolved by blue Sepharose. Alternatively, this result may suggest the removal of a protein(s) component of a multisubunit complex responsible for dCTP transport. Furthermore, there is a large increase in the total activity recovered in the HPT eluate (i.e. 965%) relative to the starting material. The recovery of activity greater than the starting material may reflect the removal of an inhibitor or interfering activity. Further studies will be required to define the role of these factors in the above observations.

Hydroxypatite chromatography is important because it offers a large single purification step common to mitochondrial anion transporters (17–23). Under the conditions described under "Experimental Procedures," the reconstituted protein eluate after HPT chromatography contained atracylodsine-sensitive AAC activity in addition to dCTP transport activity (data not shown). To improve the purification of the dCTP carrier activity, the protein eluate after HPT chromatography was applied to a blue Sepharose column to remove the AAC activity described under "Experimental Procedures." Table II shows that after HPT and blue Sepharose chromatography the internalized ADP as a counterion did not stimulate ATP uptake in reconstituted proteoliposomes. This is in contrast to HPT protein eluates reconstituted into proteoliposomes, where internalized ADP dramatically increased ATP uptake (data not shown). Furthermore, ATP uptake in proteoliposomes was observed after HPT and blue Sepharose chro-

| TABLE I | Partial purification of the dCTP carrier activity from human acute lymphocytic leukemia mitochondria |
|---------|------------------------------------------------------------------------------------------------------------------|
|          |                                                                                                                    |
| Total protein | dCTP uptake |
| Triton X-114 extract | 96 | 2.8 |
| Detergent phase | 50 | 18 |
| Aqueous phase | 33 | <1 |
| HPT eluate | 5.2 | 499 |
| Blue Sepharose eluate | 3.8 | 248 |

**Assay Conditions for dCTP Uptake**—All measurements of nucleotide uptake into reconstituted proteoliposomes were carried out at 37 °C. Transport was initiated by the addition of 4 μl of radiolabeled substrate (typically [α-32P]dCTP, 10 μM final concentration, 30 Ci/mmol) to 190 μl of proteoliposomes in a final volume of 200 μl. For competition assays, competitor was added to the desired concentration to the proteoliposome mix before the radiolabeled substrate. After the desired incubation time, the reaction mixture was placed over a Dowex ion exchange column (1 × 3.5 cm) equilibrated with 20 mM Hepes (pH 7.0) at 4 °C in order to remove the external radioactivity not transported into proteoliposomes. The liposomes were eluted with 4 ml of ice-cold equilibration buffer, collected in 16 ml of scintillation fluid, vortexed, and counted. Subtracting values obtained when a 100-fold molar excess of nonradio-labeled substrate was added 2 min prior to the radiolabeled substrate made corrections for nonspecific uptake of radioactivity. For efflux experiments, external radioactivity was removed by passing the samples through a Dowex column and collecting and counting the liposomes as described above. The transport activity was calculated by subtracting the experimental values from the control values (efflux at 4 °C).
Three separate experiments are presented. The addition of radiolabeled substrate as indicated. Means and S.D. values in the presence of 10  
M Mg\textsuperscript{2+} was obtained and reconstituted as described under “Experimental Procedures.” For ATP/ADP exchange reactions, proteins were reconstituted in the presence of 100 μM ADP. Uptake reactions were initiated by the addition of 10 μM [α\textsuperscript{32P}]ATP. dCTP was added 2 min prior to the addition of radiolabeled substrate as indicated. Means and S.D. values from three separate experiments are presented.

| Internal nucleotide | Competing nucleotide | ATP uptake Activity |
|---------------------|----------------------|---------------------|
| None                | None                 | 113 ± 3 100%        |
| 10 μM dCTP          | None                 | 63 ± 3 56%          |
| 50 μM dCTP          | None                 | 45 ± 3 40%          |
| ADP                 | 10 μM dCTP           | 50 ± 1 44%          |
| ADP                 | 50 μM dCTP           | 53 ± 3 46%          |
| ADP                 | 10 μM dCTP           | 37 ± 4 33%          |

The effect of protein concentration on the reconstitution of dCTP transport activity was investigated by progressively increasing the protein concentration in the reconstitution mix while keeping the lipid concentration constant (55 mg/ml). In reconstituted proteoliposomes, the activation effect of Mg\textsuperscript{2+} was much less pronounced than the increase in the rate of protein-mediated uptake. From these experiments, we selected 0.3 mg/ml protein as an optimum condition to characterize dCTP uptake.

Increasing the protein concentration from 0.05 to 0.4 mg/ml resulted in a parallel increase in the transporter-mediated uptake of [32P]dCTP (Fig. 2A). There also was a slight increase in the background rate of uptake with increasing protein concentration (<2 pmol/min at 0.4 mg/ml protein), but this increase was much less pronounced than the increase in the rate of protein-mediated uptake. From these experiments, we selected 0.3 mg/ml protein as an optimum condition to characterize dCTP uptake.

In Fig. 2B the dCTP uptake catalyzed by mitochondrial protein reconstituted into liposomes has been plotted as a function of time. There is an apparent initial rapid component occurring within 1 min and a slower uptake component that reached a

![Fig. 1. Optimization of dCTP uptake.](image-url)
Proteoliposomes were prepared from the protein eluate after HPT and blue Sepharose chromatography as described under “Experimental Procedures.” Vesicles were incubated with [32P]dCTP in the presence of 20 mM HEPES (pH 7.0), 2 mM CaCl2, and 0.2 mM EDTA. A illustrates the effect of protein concentration on the uptake of dCTP by proteoliposomes. Uptake was initiated by the addition of 10 μM [α-32P]dCTP for 1 min. The protein concentration of the initial reconstitution mixture was varied from 0.05 to 0.4 mg/ml. B illustrates the time- and temperature-dependent uptake of [α-32P]dCTP by proteoliposomes reconstituted with 0.3 mg/ml protein. Uptake was initiated by the addition of 10 μM [α-32P]dCTP. Vesicles were incubated for the indicated times at 37 °C (●) or 4 °C (○). Proteoliposomal uptake of [α-32P]dCTP is plotted as pmol of [α-32P]dCTP accumulated per milligram of proteoliposomal protein. C illustrates the time-dependent efflux of dCTP in proteoliposomes (reconstituted with 0.3 mg/ml protein) loaded with 20 μM [α-32P]dCTP as described under “Experimental Procedures.” Transport was initiated by incubation at 37 °C. D illustrates the time- and temperature-dependent uptake of [γ-32P]dCTP by proteoliposomes reconstituted with 0.3 mg/ml protein. Uptake was initiated by the addition of 20 μM [γ-32P]dCTP and the vesicles incubated for the indicated times at 37 °C (●) or 4 °C (○).

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steady state within 5–10 min. A temperature dependence in dCTP uptake is also observed with negligible [32P]dCTP uptake when the proteoliposomes are incubated at 4°C. Subsequent experiments were conducted at 1 min to assess the initial uptake of dCTP in reconstituted proteoliposomes. Because the insertion of protein in the lipid vesicle is in a random orientation, unidirectional transport in both directions may explain the time-dependent change in the transport rate. To measure the influx of dCTP, proteoliposomes were preloaded with [α-32P]dCTP. Fig. 2C illustrates a time-dependent efflux of [α-32P]dCTP from proteoliposomes similar to that observed for influx. To directly address if dCTP is transported without prior breakdown, dCTP was radiolabeled with 32P in the γ-position only and used for uptake studies. Fig. 2D illustrates the time- and temperature-dependent uptake of [γ-32P]dCTP. Under these conditions, [γ-32P]dCTP uptake decreases in the presence of excess nonradiolabeled dCTP. However, a 10-fold molar excess of inorganic phosphate has no effect on [γ-32P]dCTP uptake, suggesting that protein-mediated uptake of [32P]-labeled inorganic phosphate (e.g. the phosphate transporter) plays no role in the observed transport activity. Thus, it is unlikely that dCTP is broken down before uptake into proteoliposomes. In addition, the uptake of dCTP is broken down before uptake into proteoliposomes. In addition, the exchange of a counterion (including dCDP or ADP) for dCTP in reconstituted proteoliposomes, similar to that for ATP and ADP in the AAC nucleotide transport system, has not been observed.2

We have thus far demonstrated the transport activity of reconstituted mitochondrial protein by measuring the uptake of radioactive substrate into proteoliposomes. With this assay method, the amount of substrate taken up can be measured, but it is not possible to obtain precise initial kinetic parameters because the radioactive substrate remaining outside the proteoliposomes has to be removed before the radioactive substrate transported inside the vesicles can be measured. Further studies were performed to elucidate a dose-response curve for dCTP uptake in proteoliposomes. The value obtained from a Lineweaver-Burk plot (Fig. 3) indicated an apparent Km of 3 μM. The inhibition of dCTP uptake in proteoliposomes by ATP was also studied. Fig. 3 demonstrates that ATP competitively inhibits dCTP uptake. The Ki value was estimated to be 8 μM by a replot of the slopes versus the ATP concentration. Numerous studies in cell culture, particularly that of the T-lymphoblastic cell line CEM, have demonstrated dCTP intracellular pool sizes ranging from 8 to 38 μM (26–30). Whereas CEM cells are rapidly dividing cells in culture, another study using peripheral blood lymphocytes reported a dCTP pool size of 1.5 μM in quiescent cells and 18 μM in phytohemagglutinin-activated lymphocytes (31). Thus, the mitochondrial dCTP transport activity reported herein may be significant within the range of these reported intracellular dCTP concentrations.

The substrate specificity of this novel mitochondrial carrier activity was examined by investigating the ability of dCTP and its derivatives to inhibit uptake of the unidirectional, inward flux of 10 μM [32P]dCDP (Table III). If [32P]dCTP were broken down to [32P]dCDP or [32P]dCMP for transport, we would expect excess dCDP or dCMP to decrease uptake by dilution of the radiospecificity of [32P]dCDP or [32P]dCMP derived from the action of phosphatase present in the preparation. Excess unlabeled dCTP is included as a positive control. Uptake of [32P]dCTP was most affected by the triphosphate of deoxyctydine (dCTP), while the diphosphate form (dCDP) exhibited a moderate effect. In contrast, the nucleoside deoxyctydine and its monophosphate derivative (dCMP) had little effect on [32P]dCTP uptake (<10% inhibition). Thus, the reconstituted transporter discriminates between different phosphate forms of deoxyctydine. The uptake of α- and γ-labeled [32P]dCTP into proteoliposomes and the effect of excess dCDP and dCMP on uptake suggests that dCTP is the primary substrate used for transport.

E. G. Bridges, Z. L. Jiang, and Y. C. Cheng, unpublished observations.
the presence of 20 mM Hepes (pH 7.0), 2 mM CaCl₂, and 0.2 mM EDTA. Proteoliposomes (0.3 mg/ml protein) were prepared after HPT and blue Sepharose chromatography as described under "Experimental Procedures." Transport was initiated by the addition of 10 μM \([\alpha\text{-}^{32}\text{P}]\text{dCTP}\) to proteoliposomes in a buffer containing 20 mM Hepes (pH 7.0), 0.2 mM EDTA, and 2 mM CaCl₂. The concentration of competitors was 100 μM. Phosphate was added at a concentration of 5 mM. Means and S.D. values from three experiments in duplicate are presented.

Additive | dCTP uptake | Activity remaining %
---|---|---
None | 190 ± 3 | 100%
dCTP | 13 ± 2 | 7%
dCDP | 122 ± 11 | 64%
dCMP | 177 ± 2 | 93%
dCD | 200 ± 8 | 105%
Atractyloside | 285 ± 7 | 150%
N-Ethylmaleimide | 162 ± 8 | 85%
PO₄⁻ | 158 ± 12 | 83%

**Table IV**

Effect of nucleoside triphosphates on dCTP transport activity

The protein eluate from HPT and blue Sepharose chromatography was reconstituted into phospholipid vesicles as described under “Experimental Procedures.” Transport was initiated by the addition of 10 μM \([\alpha\text{-}^{32}\text{P}]\text{dCTP}\) and one of the competing nucleotides. Means and S.D. values from three separate experiments are presented. The control value of dCTP uptake was 189 pmol/min/mg protein.

| Competing nucleotide | 10 μM concentration | 50 μM concentration |
|---|---|---|
| None | 100 ± 7 | 100 ± 7 |
| dCTP | 45 ± 1 | 17 ± 2 |
| TTP | 77 ± 6 | 47 ± 1 |
| dGTP | 63 ± 5 | 32 ± 8 |
| dATP | 65 ± 3 | 36 ± 3 |
| ATP | 71 ± 7 | 32 ± 3 |
| CTP | 74 ± 1 | 50 ± 3 |
| UTP | 89 ± 2 | 73 ± 1 |
| GTP | 34 ± 1 | 20 ± 2 |

The effects of other nucleoside triphosphates on \([^{32}\text{P}]\text{dCTP}\) uptake are shown in Table IV. At equimolar concentrations, the purines GTP, ATP, dATP, and dGTP moderately reduced \([^{32}\text{P}]\text{dCTP}\) uptake in proteoliposomes with more potent inhibi-

![Fig. 3. Interaction of dCTP with reconstituted proteoliposomes and inhibition by ATP.](image)

The protein eluate from HPT and blue Sepharose chromatography was reconstituted into phospholipid vesicles as described under “Experimental Procedures.” Transport was initiated by the addition of \([\alpha\text{-}^{32}\text{P}]\text{dCTP}\) at the indicated concentrations for 1 min in the presence of 20 mM Hepes (pH 7.0), 2 mM CaCl₂, and 0.2 mM EDTA. The data are presented as a double-reciprocal plot of dCTP uptake. Velocity is expressed as pmol of dCTP uptake/min/mg of protein. The figure illustrates the dependence of the rate of dCTP uptake in proteoliposomes on substrate concentration and the inhibition of dCTP uptake in reconstituted proteoliposomes by ATP. Vesicles were incubated with \([\alpha\text{-}^{32}\text{P}]\text{dCTP}\) in the presence of ATP. Velocity is expressed as pmol of dCTP uptake/min/mg of protein. Concentrations of ATP were 0 (●), 5 (○), 10 (■), and 20 (□) μM. The Kᵢ value for ATP was determined by replot of the slopes as shown in the inset. These data represent the means and S.D. values of at least three experiments in duplicate.

The sensitivity of the reconstituted carrier to externally added inhibitors was investigated using the sulphydryl reagent N-ethylmaleimide (a potent inhibitor of the phosphate carrier) (32), atractyloside (a specific inhibitor of the AAC) (33), and phosphate. N-Ethylmaleimide, atractyloside, and phosphate had no significant inhibitory effect on dCTP transport (Table III). However, a 50% increase in dCTP uptake occurs in the presence of atractyloside. The mechanism by which atractyloside increases dCTP uptake is unclear. These data indicate that the phosphate carrier and the AAC do not play a role in the dCTP uptake activity observed in this study. We have not assessed the uptake of other dNTPs, although it is possible that there are other carrier activities for these metabolites. To our knowledge, this is the first report of a preparation of a mitochondrial dCTP carrier activity in functional form from any source.

This transport activity suggests an important role of bioactive endogenous dNTP uptake in mitochondria. Thus, cytoplasmic pools of dNTPs may be available to mitochondria, which
are continually being damaged, and for replication of their DNA. The transport activity reported in this study may also be important in mediating the mitochondrial accumulation of cytotoxic nucleoside analogs used in chemotherapy. Clinical and laboratory findings have focused attention on damage to mitochondrial function as a mechanism of toxicity of various drugs. Indeed, available evidence supports the hypothesis that the myopathy induced by anti-human immunodeficiency virus nucleoside analogs is due to inhibition of mitochondrial DNA synthesis (34, 35). The existence of the mitochondrial dCTP carrier activity described in this study may provide insight into the molecular mechanism underlying nucleoside analog-induced mitochondrial toxicity and protection.

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