Acta Medica Okayama

Volume 30, *Issue* 3 1976 *Article* 1

JUNE 1976

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Abstract

Phosphate-binding protein(s) was found in the inner mitochondrial membrane of calf heart by Sephadex G-200 and G-25 gel filtration. The binding activity was inhibited by N-ethylmaleimide and competed by a large amount of cold phosphate. The amount of phosphate bound to the fraction was 29 nmoles per mg of protein. Affinity chromatography with phosphate-bound Sepharose 4B confirmed the presence of phosphate-binding protein(s) in the active fraction of mitochondrial membrane fractionated by gel filtration.

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*PMID: 136868 [PubMed - indexed for MEDLINE]

Acta Med. Okayama 30, 147—152 (1976)

THE PRESENCE OF PHOSPHATE-BINDING PROTEIN IN INNER MITOCHONDRIAL MEMBRANE

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Received for publication, March 1, 1976

Abstract. Phosphate-binding protein(s) was found in the inner mitochondrial membrane of calf heart by Sephadex G-200 and G-25 gel filtration. The binding activity was inhibited by N-ethylmaleimide and competed by a large amount of cold phosphate. The amount of phosphate bound to the fraction was 29 nmoles per mg of protein. Affinity chromatography with phosphate-bound Sepharose 4B confirmed the presence of phosphate-binding protein(s) in the active fraction of mitochondrial membrane fractionated by gel filtration.

In mitochondria, ion translocation is performed by mechanisms, such as exchange diffusion, carrier systems and ionophores. Na^+ and K^+ are considered to be transported by ionophore mechanisms (1-3). In Ca^{2+} translocation a macromolecular carrier system was proposed by Lehninger (4), and a redox loop was postulated by Mitchell (5) for proton transport. In anion translocation many experimental results strongly suggest the existence of carrier systems (6-11), but no direct evidence has demonstrated the existence of such systems, even parts of the systems in mitochondria.

Phosphate transport in mitochondria has been investigated in energized and non-energized conditions of mitochondria from changes in light scattering and rapid centrifugation techniques, but reports on the isolation of phosphate-binding protein(s) as a part of the translocation systems are very rare.

In this communication we shall report data to show the presence of possible binding protein(s) for translocation of inorganic phosphate in inner mitochondrial membrane by using affinity chromatography.

MATERIALS AND METHODS

Calf heart mitochondria were prepared by the method of Crane, Glenn and Green (12), as described by Hatefi and Lester (13). Sonicated submitochondrial particles (C-ETP_H) were prepared by the method of Hansen and Smith (14).

C-ETP_H (90 mg of protein) were treated with STD medium containing 1.0% sodium dodecyl sulfate, 50 mM Tris-Cl, pH 7.5, and 0.2 mM dithiothreitol

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(DTT) at room temperature for 30 min.

First fractionation of treated C-ETP_H was performed at room temperature by passage through a column (2.6×85 cm) of Sephadex G-200 conditioned with STD.

Assay of the binding of inorganic phosphate (Pi) was performed at room temperature by using a column (1.0×43 cm) of Sephadex G-25 conditioned with STD medium, and ^{32}P (10 μCi) was used for the assay with or without cold Pi, sodium arsenate, or N-ethylmaleimide (NEM). The condensed sample (1.7 mg protein) which was a mixture of fractions from No. 20 to 22 (Fig. 1) was charged

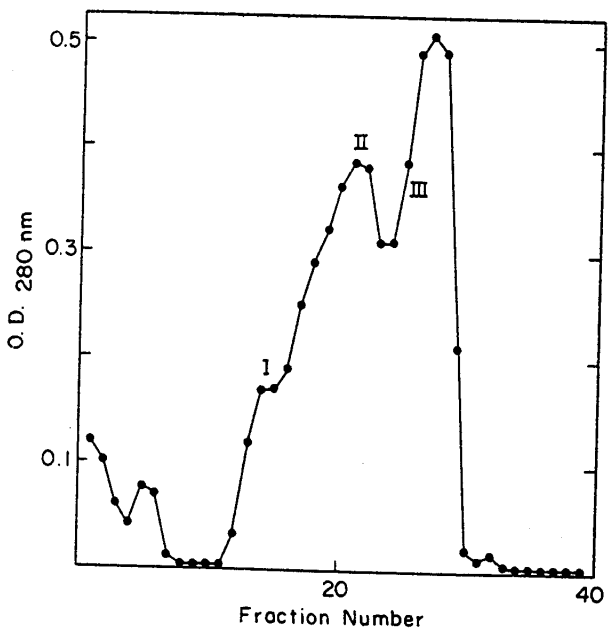

Fig. 1. Sephadex G-200 column chromatography (2.6×85 cm) of submitochondrial particles (90 mg of protein) treated with the STD medium at room temperature for 30 min. Elution was performed with the same medium, and fractions of 10.5 ml were collected.

on the column of Sephadex G-25 conditioned with STD medium and fractionated for the assay of Pi-binding.

Phosphate-bound Sepharose 4B was prepared by the method of Cuatrecasas (15) modified as follows: Sepharose 4B (10 ml packed volume) was activated with 3.2 g of cyanogen bromide at 0°C and pH 11 for 30 min, and the activated Sepharose was allowed to react with hexamethylenediamine (20 nmoles) at 4°C and pH 10 for 16 hours, and the aminohexyl derivative was treated with succinic anhydride (10 mmoles) at 4°C and pH 6 for 6 hours to prepare succinylaminohexyl derivative. The chain-elongated derivative was coupled with phosphorylethanolamine (1 mmole) in the presence of 2 mmoles of 1-ethyl-3-(3-dimethyl-

aminopropyl) carbodiimide (water soluble carbodiimide) at 20°C and pH 5 for 20 hours.

For affinity chromatography, 3.2 mg of the sample was charged on the column (1.0×12.7 cm) of Pi-bound Sepharose 4B conditioned with Tris-DTT medium containing 50 mM Tris-Cl, pH 7.5 and 0.2 mM DTT. The adsorbed material was eluted in two steps: the first step with the Tris-DTT medium and the second with potassium phosphate (0.1 M, pH 7.4).

RESULTS AND DISCUSSION

Submitochondrial particles (C-ETP$_H$) were treated with STD medium at room temperature and fractionated by gel filtration in a column of Sephadex G-200, and three peaks were separated (Fig. 1). The 2nd peak (No. 20–22 in Fig. 1) was assayed for Pi binding by the method of gel filtration in columns of Sephadex G-25. Binding activity was demonstrated in fractions No. 6 and 7 (Fig. 2A). The amount of phosphate bound to fraction No. 6 was 29 nmoles per mg of protein. The binding was not so sensitive to SDS treatment under these experimental conditions. The bound ^{32}P was diluted (competed) by cold

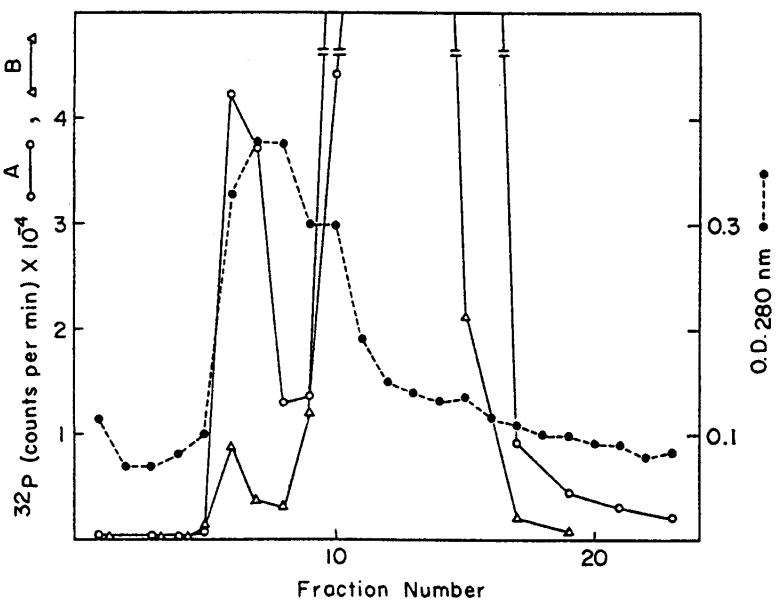

Fig. 2. Gel-filtration assay for Pi binding with a column (1.0×43 cm) of Sephadex G-25 equilibrated with STD medium. The condensed sample (1.7 mg of protein) from the 2nd peak in Fig. 1 was charged and eluted with the same medium. The volume of fractions was 1.6 ml. ^{32}Pi at 10 μCi with (B) or without (A) cold Pi (1 mM) was mixed with the sample before charging. Cold Pi was mixed with the sample 10 min before addition of ^{32}P.

Pi (Fig. 2B), and inhibition of Pi binding by arsenate which is an analogue anion of Pi was less effective (Fig. 3B and C). These findings suggest that the Pi binding protein(s) recognizes a specific conformation of Pi.

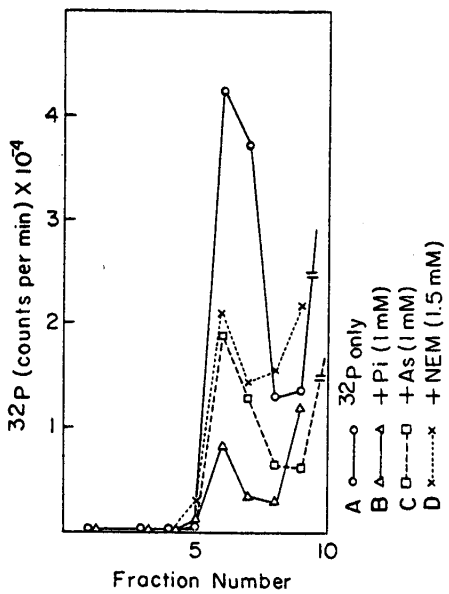

Fig. 3. Inhibition of Pi binding to protein(s) by cold Pi (B), arsenate (C) and NEM (D). The experimental conditions were the same as in the legend of Fig. 2, except for the presence of arsenate (1 mM) or NEM (1.5 mM) in system (C) and (D), respectively. The fractionation patterns of system (A) and (B) were copied from Fig. 2 to compare the effects of arsenate and NEM. Fractionation patterns after tube No. 11 were omitted.

The presence of Pi binding protein in the 2nd peak (Fig. 1) is consistent with the existence of a protein that is specifically alkylated by NEM and competitively protected by high levels of Pi from alkylation by NEM. High levels of Pi compete the inhibitory effect of NEM on energy-dependent Pi transport in mitochondria (16, 17). The 1st peak of Fig. 1 also showed weak Pi binding capacity.

The binding of Pi was also inhibited by NEM, but inhibition was not complete (Fig. 3, D). This is also consistent with the results that specificity of NEM effects on Pi transport is limited to the energy-dependent Pi uptake in mitochondria (17, 18), and we used a solubilized simple fraction in non-energized condition.

To confirm the binding of Pi to the protein(s) in the 2nd peak, phosphate-bound Sepharose 4B affinity chromatography was used. Fig. 4 clearly shows

the presence of Pi binding protein(s) in the inner mitochondrial membrane. The observed Pi binding is not attributable to accumulation of Pi into the sub-mitochondrial vesicles, which was absent in these fractions by electron microscopy (not shown).

The presence of a Pi-binding protein was shown in G-200 fractions of inner mitochondrial membrane solubilized with SDS. Whether the Pi-binding protein(s) has any enzymatic activities and performs translocation activity in Pi transport system *in vivo* are problems to be elucidated.

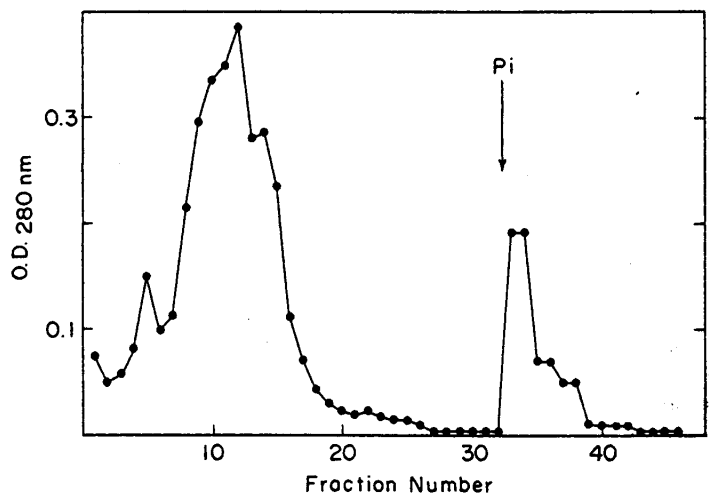

Fig. 4. Affinity chromatography of the sample from the 2nd peak in Fig. 1 with a Pi-bound Sepharose-4B column (1.0×12.7cm). The condensed sample (3.2 mg of protein) was charged on the column and was eluted first with Tris-DTT medium, and the second step elution was performed with potassium phosphate (0.1 M), pH 7.4, after completion of protein elution in the 1st step (No. 32).

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