ON THE POSSIBLE ROLE OF PHOSPHOPROTEINS AS IRON CARRIERS

A. DONELLA, L.A. PINNA and V. MORET
Institute of Biological Chemistry, University of Padova and *Centro per lo Studio della Fisiologia dei Mitocondri del C.N.R., Padova, Italy*

Received 28 July 1972

1. Introduction

Recently we have described the isolation from rat liver cytosol of a phosphoprotein fraction containing both phosphorylserine and phosphorylthreonine, which displays the same enzyme specificity as phosvitin, being phosphorylated by cytosol phosvitin kinase and dephosphorylated by intramitochondrial phosvitin phosphatase [1, 2].

2. Results

The chemical analysis of purified cytosol phosphoproteins disclosed further similarities with phosvitin, i.e. a very high content of phosphate residues and of protein-bound Fe (see table 1). The amount of iron bound to phosvitin depends on the preparation procedure [3]: when prepared according to Mecham and Olcott [4] phosvitin exhibits an iron content fairly different from that of cytosol phosphoproteins. However both phosphoproteins when exposed to an excess of $FeCl_3$ reach the same saturation level corresponding to a Fe/P molar ratio of about 0.5.

The effect of several conditions on the stability of [^{59}Fe]–phosvitin complex is shown in table 2. It can be seen that only unlabelled Fe^{3+} promotes a massive release of ^{59}Fe, as expected through a mechanism of isotopic dilution. Among the remaining di- and trivalent cations tested, a slight release of ^{59}Fe was observed only in the presence of high Co^{2+} concentrations, the remaining cations being quite ineffective, even those, like Mn^{2+}, Mg^{2+} and protamine, which bind to phosvitin through electrostatic forces resulting in insoluble complexes. Very high ionic strength (2 M NaCl) was also ineffective on the [^{59}Fe]–phosvitin linkage, while extremely acid conditions, like 1 N HCl, which are expected to prevent the ionization of the acidic groups of phosvitin, promoted only a limited release of bound ^{59}Fe.

All these findings indicate that the binding of Fe^{3+} ions to phosvitin is very tight and occurs through covalent bonding rather than through electrostatic forces.

The intracellular compartmentation of phosvitin kinase and phosphatase [8, 9] and the detection of iron bound to cytosol phosphoproteins prompted us to put forward the working hypothesis that cytosol phosphoproteins, the role of which is still obscure, might be involved in the translocation of iron across the mitochondrial membrane.

To test the validity of such an hypothesis the effect of enzymatic dephosphorylation by mitochondrial protein phosphatase on the iron binding capacity has been investigated. Owing to the great difficulty to prepare large amounts of cytosol phosphoproteins, phosvitin has been used in such experiments.

As shown in fig. 1 phosvitin phosphatase purified from rat liver mitochondria promoted quite parallel P_i and Fe release from Fe–phosvitin complex. Both the dephosphorylation and Fe release exhibit the same K_m (= 0.27 mg phosvitin/ml). Molybdate, a well known inhibitor of protein phosphatase [10] prevents both processes.

3. Discussion

The above results suggest that cytosol phosphoproteins can be looked upon at as iron carriers. On this

Table 1
Phosphate and iron content of cytosol phosphoproteins.

			Upon saturation with Fe^{3+}:	
	P (nmoles/mg)	Fe (nmoles/mg)	Fe (nmoles/mg)	Molar ratio Fe/P
Cytosol phosphoproteins	1,380	250–400*	660	0.47
Phosvitin	2,870	54	1,420	0.49
Ovalbumin	50	0.0	0.0	0.00

* Iron content was found to be fairly variable between these two figures in different preparations of cytosol phosphoproteins.

Phosphoproteins were purified by submitting to gel electrophoresis in 7.5% polyacrylamide, pH 8.5, the phosphoprotein fraction obtained from rat liver cytosol by the procedure previously described [2]: the ^{32}P-labelled protein was eluted, submitted to Sephadex G-25 gel filtration and analyzed for its phosphate and iron content. Protein was determined according to Folin and Ciocalteu [5]. Phosphate was determined according to Wagner [6]. Iron was determined colorimetrically as the bathophenanthroline complex extracted into isoamyl alcohol [7]. Phosvitin was prepared according to Mecham and Olcott [4]. Ovalbumin was from Merck. Saturation with iron was accomplished by incubating amounts of phosphoprotein equivalent to 0.1 μmoles phosphate in the presence of increasing concentrations (0.1 to 0.2 mM) of $FeCl_3$. Phosphoprotein was removed by precipitation in ice cold 15% trichloroacetic acid and analyzed for their phosphate and iron content after two washings with 15% trichloroacetic acid.

matter it must be recalled that phosvitin has been suggested to act as iron carrier during the development of chick embryo [3]. It is quite conceivable that the release of iron from the cytosol phosphoproteins involves the protein phosphatase located inside mitochondria [9]. It must be underlined that crude cytosol, lacking phosvitin phosphatase, has been found to be unable to release Fe from phosvitin.

It seems reasonable therefore to assume that cytosol phosphoproteins, once phosphorylated by cytosol kinase, bind Fe ions which are released by protein phosphatase inside mitochondria.

Several findings are fitting with such a scheme: i) The high affinity of cytosol phosphoproteins for iron; ii) The strictly intramitochondrial compartmentation of phosvitin phosphatase which releases P_i and Fe^{3+} in the same ratio as they are bound to the protein; iii) The specific inhibition of intramitochondrial enzyme by Fe ions, which can be looked at as a kind of feedback regulation [8]; iv) The low molecular weight of cytosol phosphoproteins, under 9,000, which makes their entrance through the outer mitochondrial membrane quite expectable.

Moreover, although the mechanism of iron uptake by mitochondria is still unknown, from Garland's experiments on *T. utilis* mitochondria [13] it can be

Table 2
Effect of different conditions on the stability of [^{59}Fe]–phosvitin complex.

	Phosvitin bound [^{59}Fe] (cpm)
Control	2826
Plus 1 mM $FeCl_3$	710
Plus 0.1 M $Co(NO_3)_2$	2690
Plus 1 M $Co(NO_3)_2$	2530
Plus 0.1 M $MnSO_4$	2850
Plus 0.1 M $ZnCl_2$	2810
Plus 0.1 M $CuSO_4$	2775
Plus 0.1 M $LaCl_3$	2780
Plus 0.1 M $MgCl_2$	2820
Plus 0.1 M $CaCl_2$	2802
Plus 2.0 M NaCl	2800
Plus 1.0 N HCl	2214
Plus Protamine, 1 mg/ml	2840

^{59}Fe-labelled phosvitin was prepared by incubating 40 mg of phosvitin in 2 ml of 1.0 mM $FeCl_3$ containing 4 μCi as ^{59}Fe. After 2 hr at room temp. the sample was filtered through a Sephadex G-25 column (1.8 × 36 cm) equilibrated with distilled water. Labelled phosvitin, eluted at the V_0, was lyophilyzed.

0.2 mg of [^{59}Fe]–phosvitin were incubated in a total volume of 1.0 ml under the conditions indicated. After 15 min at room temp., [^{59}Fe]–phosvitin was precipitated by addition of 0.2 ml 50% trichloroacetic acid, washed 3 times with 10% trichloroacetic acid and counted in a thin window Geiger counter.

deduced that extramitochondrial components seem to be required for such an uptake.

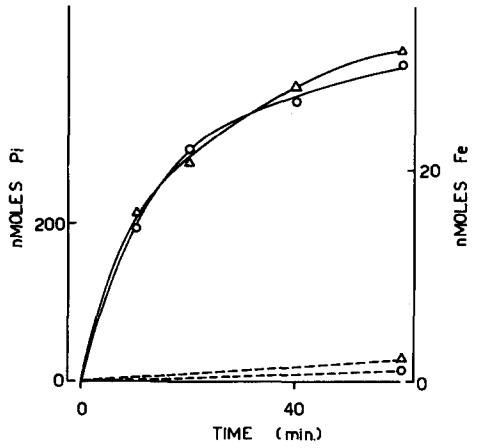

Fig. 1. P_i and Fe^{3+} release from phosvitin catalyzed by mitochondrial phosvitin phosphatase. Fe–phosvitin complex was prepared by adding slowly 0.4 ml of 0.1 M $FeCl_3$ to 5 ml of a 2% phosvitin solution at neutral pH by continuous stirring. Small amounts of insolubilized protein were discarded by centrifugation and the clear supernatant was dialyzed for 48 hr against 3 changes of distilled water. The resulting Fe–phosvitin complex was about 20% saturated with Fe (Fe/P = 0.1).

0.35 mg of the Fe–phosvitin complex was incubated at 37° for the times indicated in 1.0 ml of a medium containing: 100 mM acetate buffer pH 5.0; 6.2 mM cysteine and 20 μg of purified mitochondrial phosvitin phosphatase [11]. Controls were run omitting phosphatase. Reaction was stopped by addition of 0.3 ml of 50% trichloroacetic acid. After cooling, the precipitate was discarded by centrifugation and 50 mg of Dowex 50 (H^+) × 8 were added to the supernatant. P_i was determined on the resin supernatant according to Martin and Doty [12]. Fe^{3+} was eluted from the resin by addition of 5 M HCl (0.6 ml) and determined as described in table 1. (o—o—o) P_i released; (o- -o- -o) P_i released in the presence of 1.0 mM Na_2MoO_4; (Δ—Δ— Δ) Fe^{3+} released; (Δ- -Δ- -Δ) Fe^{3+} released in the presence of 1.0 mM Na_2MoO_4.

References

[1] L.A. Pinna, G. Clari, V. Moret and N. Siliprandi, FEBS Letters 5 (1969) 77.
[2] L.A. Pinna, G. Clari and V. Moret, Biochim. Biophys. Acta 236 (1971) 270.
[3] O. Greengard, A. Sentenac and N. Mendelsohn, Biochim. Biophys. Acta 90 (1964) 406.
[4] D.K. Mecham and H.S. Olcott, J. Am. Chem. Soc. 71 (1949) 3670.
[5] O. Folin and V. Ciocalteu, J. Biol. Chem. 73 (1927) 627.
[6] H. Wagner, Fette Seifen 62 (1960) 1107.
[7] K.A. Doeg and D.M. Ziegler, Arch. Biochem. Biophys. 97 (1962) 37.
[8] A. Donella, L.A. Pinna, V. Moret and N. Siliprandi, in: Biochemistry and Biophysics of Mitochondiral Membranes (Academic Press, New York, 1972) p. 577.
[9] G. Clari, L.A. Pinna, V. Moret and N. Siliprandi, FEBS Letters 17 (1971) 300.
[10] K. Paigan and S.K. Griffiths, J. Biol. Chem. 234 (1959) 299.
[11] G. Magni, G. Caulini and V. Moret, Biochim. Biophys. Acta 242 (1971) 123.
[12] J.B. Martin and O.M. Doty, Anal. Chem. 21 (1949) 965.
[13] P.A. Light, C.I. Ragan, R.A. Clegg and P.B. Garland, FEBS Letters 1 (1968) 4.