THE ROLE OF METAL IONS IN THE TRANSPORT OF SUBSTRATES IN MITOCHONDRIA

S. PASSARELLA, F. PALMIERI and E. QUAGLIARIELLO
Department of Biochemistry, University of Bari, Bari, Italy

Received 7 August 1973
Revised version received 10 September 1973

1. Introduction

Tyler and Newton [1] found that bathophenanthroline sulfonate and other metal-complexing agents inhibit succinate oxidation far more with intact mitochondria than with mitochondria treated ultrasonically, and postulated that the reagents form a complex with a metal ion involved in the mechanism of succinate transport into the mitochondria.

The object of the work described in this paper is to investigate (i) whether other transporting systems, besides the dicarboxylate carrier, are inhibited by bathophenanthroline; (ii) the location of the metal ion(s) on the carrier molecules relative to the substrate-binding sites; (iii) the role played by metal ion(s) in the mechanism of substrate transport across the mitochondrial membrane. The effect of bathophenanthroline on the kinetics of anion–anion exchanges was measured directly, with the advantage of using lower concentrations of the inhibitor than is necessary when following substrate oxidation and thus avoiding secondary effects [1, 2]. The results suggest that a metal ion complexed by bathophenanthroline is located at carrier sites specific for binding dicarboxylates, tricarboxylates and 2-oxoglutarate, but that such a metal ion is not involved in the binding of P_i. It is proposed that the metal ion is involved in the binding of the carboxylic groups of these substrates. Part of this work has been communicated [3, 4].

2. Materials and methods

Bathophenanthroline sulfonate was obtained from Sigma. Other reagents were obtained as described [5, 6].

Rat liver mitochondria were isolated and loaded with P_i or malate as previously described [6]. The kinetics of anion exchanges were studied by the 'inhibitor stop method', essentially as described previously [5, 8]. Generally, mitochondria were incubated at 9°C for 1 min in 1.0 ml medium, the uptake started by adding the labelled substrate and stopped a few seconds later with an inhibitor, like phenylsuccinate or 1,2,3-benzene-tricarboxylate. After rapidly centrifuging the mitochondria, the radioactivity in the pellets was measured in a scintillation counter.

3. Results

Fig. 1A shows the effect of increasing concentrations of bathophenanthroline on the [^{14}C]malonate–P_i, [^{14}C]citrate–malate and [^{14}C]oxoglutarate–malate exchanges, which reflect the activity of the dicarboxylate, the tricarboxylate and the oxoglutarate carrier respectively. The data demonstrate that bathophenanthroline inhibits all three carriers, giving in each case half maximal inhibition at approximately the same concentration. The effects of bathophenanthroline on the P_i uptake via the P_i carrier and the dicarboxylate carrier are compared in fig. 1B. In agreement with the inhibition of the malonate–P_i exchange, bathophenanthroline also inhibits the P_i uptake via the dicarboxylate carrier. On the other hand, the P_i carrier is very little affected by bathophenanthroline at the same concentrations.

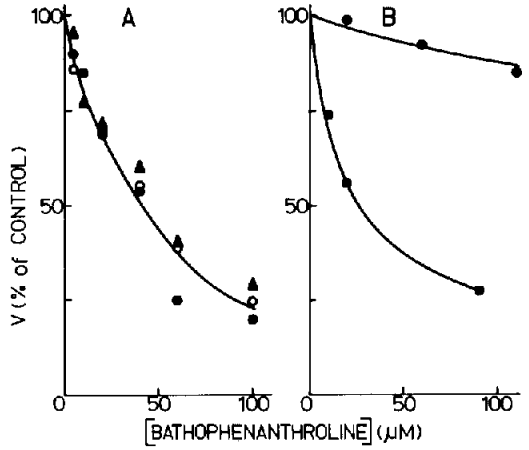

Fig. 1. The effect of increasing concentrations of bathophenanthroline on the substrate transporting system of rat liver mitochondria: A) The reaction mixture contained 100 mM KCl, 20 mM *N*-2-hydroxyethyl-piperazine-*N'*-2-ethanesulfonate (HEPES), 1 mM EGTA, pH 7.0, 1 μg rotenone and 2.6 mg protein of P_i-loaded mitochondria for exchange with malonate or 2.1 mg protein of malate-loaded mitochondria for citrate and oxoglutarate exchange. The reaction was initiated by addition of 0.25 mM [^{14}C]malonate (▲), 0.1 mM [^{14}C]citrate (●) or 30 μM [^{14}C]oxoglutarate (○). When present, bathophenanthroline was added simultaneously with the labelled substrate. Other conditions are as indicated in Materials and methods; B) Experimental conditions as in fig. 1A, except that unloaded mitochondria (2.6 mg) and either 1mM *N*-ethylmaleimide (■) to inhibit the P_i carrier or 5 mM phenylsuccinate (●) to inhibit the dicarboxylate carrier were present. The reaction was initiated by addition of 0.2 mM $^{32}P_i$.

The specificity of the interaction of the metal-complexing agent with the substrate-transporting systems was further examined by testing the ability of ions, which are known to be chelated by bathophenanthroline [9, 10], to prevent and reverse the inhibition. The data reported in table 1 show that Co^{2+} and Cu^{2+} decrease the extent of the inhibition of the dicarboxylate, the tricarboxylate and the oxoglutarate carriers, when added together with bathophenanthroline, or remove the inhibition when added after bathophenanthroline. The inhibition by bathophenanthroline was also prevented or reversed by low concentrations of Fe^{2+}, Zn^{2+} and Ni^{2+} ions, while Mg^{2+} and Mn^{2+} had no effect. The concentration dependence of the influence of Co^{2+} on the bathophenanthroline inhibition of malonate–P_i exchange is shown in fig. 2. The rate of malonate–P_i exchange is reduced 51% by 50 μM bathophenanthroline (from 33.5–16.5 μmoles/min per g protein), but only about 7% by bathophenanthroline plus 20 μM Co^{2+} in both prevention and reversal of the inhibition. It is clear that Co^{2+} ions are effective in abolishing the inhibition at a concentration lower than that of bathophenanthroline in agreement with the ability of this metal ion to complex with 1–3 molecules of bathophenanthroline [10, 11].

The inhibition of the substrate carriers by low concentrations of bathophenanthroline has been analysed in the presence of different substrate concentrations, as Lineweaver–Burk plots. As shown in figs. 3A and 3B, the addition of 40 μM bathophenanthroline together with [^{14}C]citrate of [^{14}C]oxoglutarate increases the K_m without changing the V_{max} of citrate and oxoglutarate uptake, in agreement with a competitive type of inhibition. In these experiments the K_i of bathophenanthroline for citrate uptake is 25 μM and that for oxoglutarate uptake is 30 μM. Previous work [5] has shown that the dicarboxylate carrier has two separate binding sites, one specific for P_i and the other specific for the dicarboxylates. Figs. 3C and 3D show that bathophenanthroline competitively increases the K_m of the malonate–P_i exchange without changing the V_{max}, and yields an apparent K_i of 25 μM (cf. ref. [8]). On the other hand, the bathophenanthroline inhibition of the P_i–P_i exchange is not reversed by raising the substrate concentration, i.e. is predominantly non-competitive, suggesting that the metal-complexing agent binds at the dicarboxylate-binding site and away from the P_i-binding site.

Further evidence that bathophenanthroline competes directly with di- and tricarboxylates for the same binding site on the dicarboxylate, tricarboxylate and oxoglutarate carrier was obtained by performing experiments in which the bathophenanthroline concentration was varied at fixed substrate concentration. The plots of $1/V$ against concentration of bathophenanthroline, illustrated in fig. 4, show that the curves for two different substrate concentrations are linear, indicating that bathophenanthroline is a true competitive inhibitor with respect to malonate, citrate and oxoglutarate.

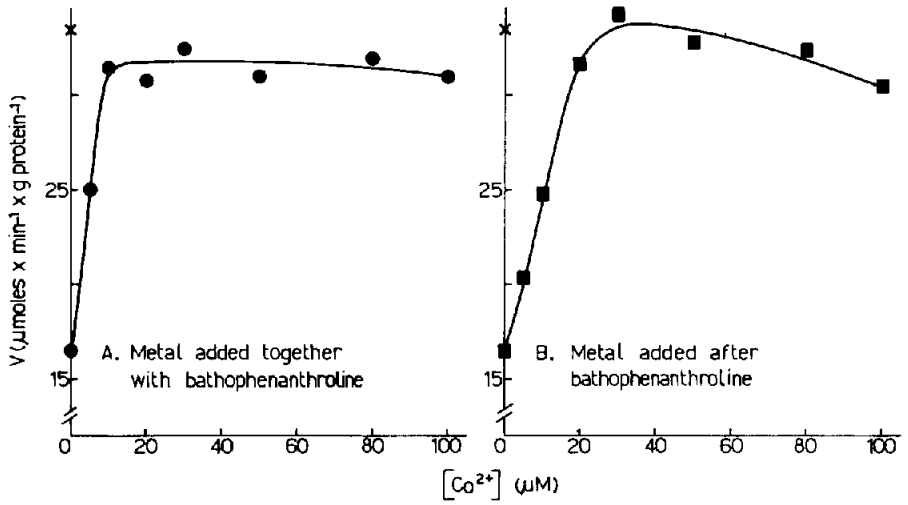

Fig. 2. The concentration dependence of cobaltous ions for the prevention and the removal of the bathophenanthroline inhibition of the malonate–P_i exchange. Experimental conditions as in table 1, except that [^{14}C]malonate was used at a concentration of 0.2 mM. The order of additions was as follows: in A, $CoCl_2$ together with bathophenanthroline after 40 sec and [^{14}C] malonate after 60 sec incubation; in B, bathophenanthroline after 40 sec, $CoCl_2$ after 50 sec and [^{14}C]malonate after 60 sec incubation. Mitochondrial protein was 2.2 mg. The symbol X on the ordinate represents the control with the addition of [^{14}C]malonate and in the absence of bathophenanthroline.

Table 1

The influence of Cu^{2+} and Co^{2+} ions on the inhibition of malonate–P_i, citrate–malate and oxoglutarate–malate exchanges by bathophenanthroline.

Additions	% Inhibition of the uptake of		
	[^{14}C]malonate	[^{14}C]citrate	[^{14}C]oxoglutarate
^{14}C-labelled substrate	0	0	0
^{14}C-labelled substrate+bathophenanthroline	50	42	33
^{14}C-labelled substrate+Cu^{2+}	4	0	3
^{14}C-labelled substrate+Co^{2+}	0	5	0
^{14}C-labelled substrate+bathophenanthroline+Cu^{2+}	12	19	5
^{14}C-labelled substrate+bathophenanthroline+Co^{2+}	13	12	0
Bathophenanthroline followed by Cu^{2+} and ^{14}C-labelled substrate	10	5	8
Bathophenanthroline followed by Co^{2+} and ^{14}C-labelled substrate	23	6	1

The reaction mixture contained 100 mM KCl, 20 mM HEPES–Tris, pH 7.0, 1 μg rotenone, 3 μg oligomycin and 1.8 mg P_i-loaded mitochondria (for malonate exchange) or malate-loaded mitochondria (2.0 mg and 1.7 mg for citrate and oxoglutarate exchange respectively). In the samples relative to lines 1–6, additions were made after 1 min incubation; in those relative to lines 7–8, bathophenanthroline was added after 40 sec incubation followed by metal ions and ^{14}C-labelled substrates after further 10 and 20 sec respectively. The following concentrations were used: 0.25 mM [^{14}C]malonate, 0.1 mM [^{14}C]citrate, 30 μM [^{14}C]oxoglutarate, 50 μM bathophenanthroline, 0.1 mM $CuSO_4$ and 0.1 mM $CoCl_2$.

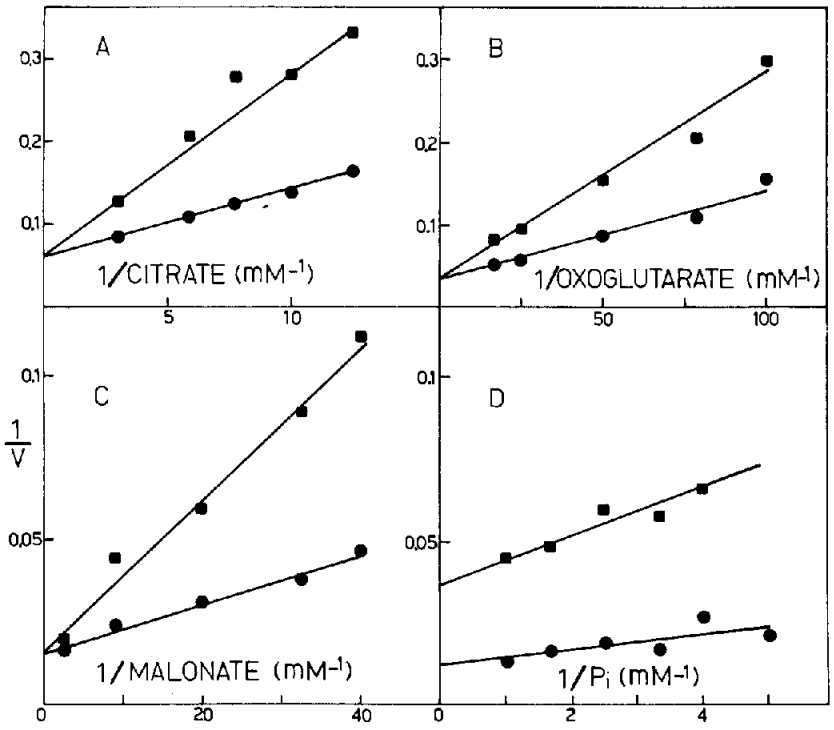

Fig. 3. Kinetic analysis of the inhibition of the citrate–malate, oxoglutarate–malate, malonate–P_i and P_i–P_i exchanges by bathophenanthroline, using the double reciprocal plot. In A and B the experimental conditions were as in fig. 1A. Mitochondral protein was 2.3 mg and the concentration of bathophenanthroline was 40 μM. In C and D the reaction mixture contained 0.215 M sucrose, 5 mM HEPES–Tris, pH 7.0, 10 mM KCl, 1 mM $MgCl_2$, 1 μg rotenone, 3 μg oligomycin, 1 mM *N*-ethylmaleimide and P_i-loaded mitochondria (1.4 mg protein in C and 2.8 mg in D). The concentration of bathophenanthroline was 40 μM in C and 17 μM in D. (●), control; (■), with bathophenanthroline.

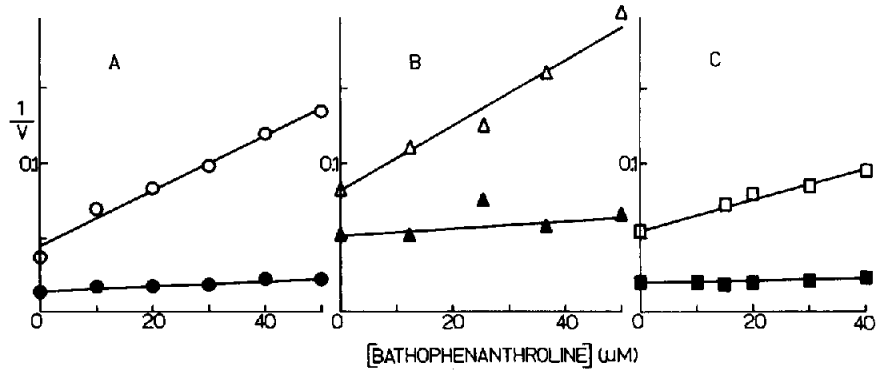

Fig. 4. Kinetic analysis of the inhibition of the malonate–P_i, citrate–malate and oxoglutarate–malate exchanges by bathophenanthroline, using the Dixon plot. In A, experimental conditions as in fig. 3C, except that bathophenanthroline was used at the concentrations indicated and [^{14}C] malonate at 2 concentrations: 0.04 mM (○) and 0.4 mM (●). In B and C, experimental conditions as in fig. 1A, except that [^{14}C] citrate was used at 0.06 mM (△) and 0.33 mM (▲), and [^{14}C]oxoglutarate at 0.017 mM (□) and 0.17 mM (■). Mitochondrial protein was 1.8, 1.4 and 1.1 mg in A, B and C respectively.

4. Discussion

The results suggest that the dicarboxylate, the tricarboxylate and the oxoglutarate carriers may contain a metal ion. Thus, the prevention of the bathophenanthroline inhibition by some metal ions is evidence that the metal-chelating properties of the inhibitor are responsible for the observed effects. The reversal of the inhibition further indicates that the postulated metallocarrier–chelate complex is dissociable, in agreement with the completely competitive nature of the inhibition with respect to the substrates. The kinetic data strongly suggest that the metal ion is located at the substrate-binding sites of the carriers which catalyze the transport of the Krebs cycle intermediates across the mitochondrial membrane. On this basis it is tentatively proposed that the metal chelates with two carboxylic groups present in all these substrates. This is in agreement with the ability of the substrates to complex with metal ions, e.g. Fe^{2+} and Cu^{2+} [12] and with the observations that (i) dicarboxylates bind to all three carriers, although with a different affinity, and (ii) the carboxylic groups must be in *cis*-configuration [3, 6, 7]. As a contrast case, it is interesting that the P_i carrier is not inhibited, and that the P_i uptake via the dicarboxylate carrier is only indirectly affected by bathopenanthroline through the formation of a ternary complex (carrier–P_i–bathophenanthroline) which is unable to transport P_i.

References

[1] Tyler, D.D. and Newton, J. (1970) FEBS Letters 8, 325.
[2] Palmer, J.M. (1970) FEBS Letters 6, 109.
[3] Quagliariello, E. and Palmieri, F. (1972) in: Biochemistry and Biophysics of Mitochondrial Membranes, p. 659, Academic Press.
[4] Passarella, S., Palmieri, F. and Quagliariello, E. (1973) 9th Intern. Congr. Biochem., Stockholm, abstr. p. 278.
[5] Palmieri, F., Prezioso, G., Quagliariello, E. and Klingenberg, M. (1971) Eur. J. Biochem. 22, 134.
[6] Palmieri, F., Stipani, I., Quagliariello, E. and Klingenberg, M. (1972) Eur. J. Biochem. 26, 587.
[7] Palmieri, F., Quagliariello, E. and Klingenberg, M. (1972) Eur. J. Biochem. 29, 408.
[8] Meisner, H., Palmieri, F. and Quagliariello, E. (1972) Biochemistry 11, 949.
[9] Schilt, A.A. (1966) Talanta 13, 895.
[10] Brandt, W.W., Dwyer, F.D. and Gyarfas, E.D. (1954) Chem. Rev. 54, 959.
[11] Yamamoto, Y., Kumamaru, T., Tsubouchi, M. and Okimura, I. (1967) Bunseky Kagazu 16, 937.
[12] Martell, A.E. (1964) in: Stability constants of metal-ion complexes p.357, The Chemical Society, London.