CONVERSION OF ISOCITRATE TO CITRATE AND ACCUMULATION OF THE CITRATES IN MITOCHONDRIA

E. J. HARRIS

Department of Biophysics, University College London, Gower Street, London WC1, England

Received 12 December 1968

1. Introduction

The equilibrium between the two citrates catalysed by aconitate hydratase (E.C. 4.2.1.3) can be used to obtain a value for the prevalent free Mg^{2+} concentration [1,2]. This is because the higher stability of the Mg-citrate complex favours conversion of more isocitrate to citrate.

When the natural enantiomorph of isocitrate is added to a suspension of mitochondria, most of it is converted to citrate and part of the latter emerges. The final ratio between the two acids in the particles presumably reflects the level of free Mg^{2+} present with the internal citrates. As more citrate is accumulated there will be less free Mg^{2+} left so the conversion ratio will fall.

Since the results indicated the presence of free Mg^{2+} even when considerable internal accumulation of citrate had occurred, it is relevant that the adenine nucleotide ratio $AMP \times ATP/ADP^2$ holding between the internal nucleotides can be far from the adenylic kinase equilibrium value. Taken with the presence of free Mg^{2+}, the maintained departure from equilibrium shows that little or no internal adenylic kinase is present (cf. ref. [3]).

2. Methods

Rat liver mitochondria were prepared by Schneider's method [4]. Homogenization was effected in 0.25 M sucrose with 1 mM EGTA and this mixture was also used for the first 2 washes. The third wash and resuspension at about 50 mg/ml was with 0.25 M sucrose. Protein was determined by a biuret method. Analyses for K and Mg were made with an atomic absorption flame spectrophotometer.

The conversion of threo-d-(+) isocitrate (Sigma) to citrate was measured in 0.2 ml samples taken successively from suspensions having 6–8 mg protein/ml at 20°. The medium contained 250 mM sucrose, 20 mM Tris chloride pH 7.4, 5 mM KCl, rotenone 1.6 μg/ml and succinate 3 mM. Trace amounts of tritiated water and ^{14}C-sucrose were also added. The successive samples were pipetted into small tubes which had been preloaded with 40 μl perchloric acid on which about 15 μl silicone oil was floated. The details have been given by Harris and Van Dam [5]. The tubes were each centrifuged for 1 min in a Coleman microfuge. The supernatant remaining over the silicone was promptly acidified. Samples of deproteinised supernatant and of the acid extract of the mitochondria recovered from beneath the silicone were assayed enzymatically for citrate and isocitrate. Samples were also used for assay of ^{14}C-sucrose and tritiated water to find how much of each was moved with the mitochondria.

The values of the adenine nucleotide contents were measured in samples withdrawn from incubations made with various additions. Samples were taken into cold perchloric acid. After deproteinisation the fluid was neutralised and used for assay of the 3 nucleotides.

The assays were made on a modified Eppendorf fluorimeter using the methods essentially as given in Bergmeyer [6].

3. Results

3.1. *Isocitrate to citrate conversion*

Values found for citrate and isocitrate in sets of 3 samples taken at 30 sec intervals after an addition were all similar, that is, the conversion was completed within 30 sec. Contents of the two acids in the medium and in the mitochondrial pellets are given in table 1. The pellet values have not been corrected for the medium carried down in the adherent water and in the sucrose-accessible space (about 3.5 ml/g in all) because the acids are sufficiently concentrated in the mitochondria to render this unnecessary.

The results show (1) that the total of the two citrates is already close to the saturation value of the mitochondrial capacity when the applied level is only 0.26 mM, (2) that the citrate/isocitrate ratio can be at least 20; emergence of the citrate tends to keep the ratio in the medium equal to that in the mitochondria, (3) taking the solvent water in the space inaccessible to sucrose as 1 ml/g protein the intramitochondrial citrate can be 100 times as concentrated as it is in the medium.

Rapid interconversion of the citrates has been shown spectrophotometrically by Chappell and Robinson [8]. The method used here only shows that the conversion has occurred within the time required to separate the first sample (30 sec).

Taken with an internal ionic strength of 0.15, provided by the K^+ salts, a citrate/isocitrate ratio of 20 corresponds to about 0.4 mM Mg^{2+} [2]. When the isocitrate added is the equivalent of a total citrate concentration of 1 mM or more the conversion to citrate is less complete and is less than the equilibrium value for a Mg-free system (which is about 8 according to Randle, Denton and England [1].

3.2. *Adenine nucleotide ratios*

The evidence that at low citrate levels there is sufficient internal Mg^{2+} to leave some in the ionic form means in turn that there must have been sufficient Mg to chelate with the internal nucleotides. Adenylic kinase, if present, would have its preferred substrate, Mg.ATP, to react with. The results of nucleotide assays (given in ref. [9]) allow calculation of the ratios $R = ATP \times AMP/ADP^2$. The value of R can undergo wide variations, from 0.3 to 8, according to the conditions of incubation of the mitochondria. Although the values found in samples of the stock mitochondrial suspension are consistent with the adenylic equilibrium in a Mg^{2+}-free system (given as 0.32 [6]) the adjustment to this value or to the higher one expected in presence of Mg^{2+} (1.0) does not occur over periods of 10 min.

Table 1

Extent of conversion of isocitrate to citrate by a suspension of rat liver mitochondria at 20°. Successive additions of isocitrate were made and the suspension was sampled; the mitochondria in the sample were separated. Values for the two citrates were obtained both for the supernatant and for an acid extract of the mitochondria. Values obtained after 30, 60 or 90 sec after a given addition showed no temporal change was taking place.

Supernatants			Extracts of mitochondria			Ratio: col. 4/col. 1 μmole/g protein over mM
(1) Citrate (C) (mM)	(2) Isocitrate (I) (mM)	(3) Ratio C/I	(4) Citrate (C) (μmole/g protein)	(5) Isocitrate (I)	(6) Ratio C/I	
0.08	0.004	20	13.0	0.46	28	160
0.26	0.015	18	26.0	1.50	17	100
0.60	0.030	20	29.5	1.93	15	50
1.01	0.054	18.5	29.0	2.3	12.5	29
1.78	0.113	15.8	27.0	4.44	6.1	15

The medium contained: sucrose 250 mM; Tric chloride pH 7.4, 20 mM; KCl, 5 mM; rotenone, 1.6 μg/ml; succinate 3 mM.

4. Discussion and conclusions

The concentration of citrate by mitochondria, already noted by Schneider, Streibich and Hogeboom [10] obviously has primary importance in cellular metabolic control because maintenance of a low cytoplasmic citrate will be associated with little inhibition of phosphofructokinase and low activation of acetyl-CoA carboxylase. The low level of cytoplasmic citrate may also limit the rate of cleavage of citrate to form acetyl-CoA and oxalacetate.

The results in table 1 show that the citrates are accumulated in mitochondria to a total level (30 μmole/g) approximating the equivalent of the mitochondrial K^+, which was 90–100 μmole/g protein in these experiments. It is still not known what anion is the major one displaced by the citrate; analyses for phosphate indicated that only a few μmole/g were moved into the medium when citrate was taken up.

There must be both rapid conversion to and egress of the citrate so other methods have to be used to obtain the kinetics [8]. The completeness of the conversion to citrate in the mitochondria even when they contain 30 μmole citrate/g protein would be consistent with their Mg (which totalled 33 μmole/g) being available to complex with citrate. On this basis other Mg^{2+} complex forming substances, such as adenine nucleotides, would be expected to form a proportion of their complexes. Adenylic kinase, for which the Mg-complexes are the preferred substrates, would be expected to be active if present at the site of the nucleotides. The fact that large displacements from the adenylic kinase equilibrium can be sustained for periods of minutes then points to the near absence of the enzyme from the mitochondrial interior. As Heldt and Schwalbach [3] have discussed it seems likely that nucleotide mono- and di-phosphokinases suffice to transfer phosphate groups between the internal nucleotides.

The extensive and rapid conversion of isocitrate to citrate, with appearance of the latter in the medium in exchange for the former, shows that discussions of isocitrate metabolism based on the total isocitrate added, for example, to a mitochondrial suspension are meaningless. The relevant concentration is doubtless that prevailing internally at the site of the enzyme. This depends on the prevalent citrate/isocitrate ratio, which these experiments show to fall as the addition of isocitrate is increased. The degree of accumulation will also depend on the metabolic activity [11] and on the levels of facilitating anions [12,13].

Acknowledgements

This work was supported by grants from the Medical Research Council, the Wellcome Trust, and the Muscular Dystrophy Association of America. Thanks are due to Miss Celia Berent for help with analyses and to Drs J.McD.Blair and E.M.Chance for advice.

References

[1] P.J.Randle, R.M.Denton and P.J.England, in: Metabolic Roles of Citrate, ed. T.H.Goodwin (Academic Press, London, 1968) p. 87.
[2] J.McD.Blair, FEBS Letters 1 (1968) 100.
[3] H.W.Heldt and K.Schwalbach, European J. Biochem. 1 (1967) 199.
[4] W.C.Schneider, J. Biol. Chem. 176 (1948) 259.
[5] E.J.Harris and K.Van Dam, Biochem. J. 106 (1968) 759.
[6] H.U.Bergmeyer, Methods of Enxymatic Analysis (Academic Press, London, 1963).
[7] E.Pfaff, M.Klingenberg, E.Ritt and W.Vogell, European J. Biochem. 5 (1968) 222.
[8] J.B.Chappell and B.H.Robinson, in: Metabolic Roles of Citrate, ed. T.H.Goodwin (Academic Press, London, 1968) p. 123.
[9] E.J.Harris, in: Round Table Discussion on Energy Level and Metabolic Control in Mitochondria, eds. E.C.Slater, J.M.Tager, S.Papa and E.Quagliarello (Adriatica Press, Bari, 1969) p. 19.
[10] W.C.Schneider, M.J.Streibich and G.H.Hogeboom, J. Biol. Chem. 162 (1956) 969.
[11] E.J.Harris, Biochem. J. 109 (1968) 247.
[12] J.B.Chappell, Biochem. J. 90 (1964) 225.
[13] S.M.Ferguson and G.R.Williams, J. Biol. Chem. 241 (1966) 3696.