IN SITU REGULATION OF YEAST CITRATE SYNTHASE. ABSENCE OF ATP INHIBITION OBSERVED *IN VITRO*

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1. Introduction

The regulation of the citric acid cycle and its component enzymes has been the subject of intensive study by many investigators. Krebs and Lowenstein [1] first concluded that the rate-limiting step of the cycle must be that catalyzed by the 'initial' enzyme, citrate synthase (EC 4.1.3.7). Hathaway and Atkinson [2] then discovered that yeast citrate synthase is strongly inhibited by ATP and subsequent reports have described similar inhibition of citrate synthase isolated from rat liver [3, 4], rat heart [4], beef heart and liver [5], pig heart [2, 6], trout liver [7] and lemon [8]. These findings have become accepted as the *in vitro* demonstration of a feedback mechanism whereby ATP, the ultimate end-product of the citric acid cycle, controls the activity of the first enzyme of the cycle, and general text-books of biochemistry now cite this as an important regulatory phenomenon.

However tempting is the extrapolation of these *in vitro* observations to the *in vivo* situation we must remain aware of the dangers inherent in so doing. Measurements of enzymic activity and its inhibition are normally performed under conditions far removed from those obtaining in the living cell. Although it may be possible to make such measurements in the presence of 'physiological concentrations' of substrates and effectors, insofar as these are known, the removal of an enzyme from the cell must inevitably destroy the particular environment it experienced within that cell. Conventional cellular disruption leads to massive dilution of cell constituents with accompanying loss of molecular interactions and both these factors may alter the properties of enzymes as they are observed in such extracts compared with their intracellular behaviour.

In a previous report [9] we suggested that the use of permeabilized bacterial cells would simulate, at least partially, the *in vivo* conditions. By destroying the cell's natural permeability barriers, e.g. by treatment with toluene, access to intracellular enzymes may be gained without total destruction of the cell. Similar proposals have been made by Reeves and Sols [10] who suggested the term '*in situ*' to describe the conditions within the permeabilized cell and Serrano et al. [11] have reported that yeast cells, like those of bacteria, may also be permeabilized by treatment with toluene, thereby permitting the examination of yeast enzymes *in situ*.

In this communication we report our studies of the behaviour of citrate synthase in permeabilized yeast cells. The results indicate that the *in situ* enzyme differs considerably from the isolated enzyme, most importantly in being insensitive to ATP inhibition. The implications for the *in vivo* control of citrate synthase are considered.

2. Experimental

Packed baker's yeast (*Saccharomyces cerevisiae*) was obtained commercially (Distillers Co. Ltd.) and permeabilized by the method previously described [9] with the modification that the toluene was introduced as a solution in ethanol [11]. The cells were first washed with 0.1 M Tris–HCl, pH 8, collected by centrifugation and resuspended in the same buffer to a concentration of 0.1 g/ml. This suspension was warmed to

37°C and 0.05 ml of toluene–ethanol (1:4 v/v) was added per ml. After vigorous mixing the suspension was kept at 37°C for 5 min and then cooled in ice. The cells were collected by centrifugation, resuspended and washed in 0.1 M Tris–HCl, pH 8, and again centrifuged. This washing and centrifugation step was repeated once more to ensure removal of toluene and low molecular weight metabolites and the toluenized cells were finally resuspended in 0.1 M Tris–HCl, pH 8, to a concentration corresponding to 0.1 g original packed yeast/ml.

For the preparation of a cell-free extract the above toluenization procedure was carried out but the final resuspension of the cells was done to a concentration corresponding to 1 g original yeast/ml. This was passed twice through a French press at 12000 lb/sq in and then centrifuged at 25000 *g* for 30 min. The supernatant solution was filtered through muslin to remove the fatty material and protamine sulphate (1 mg/10 mg protein) was added. After standing for 15 min the precipitate was removed by centrifugation and the supernatant solution (approx. 60 mg protein/ml) was used in the experiments below.

Both citrate synthase and succinate thiokinase (EC 6.2.1.4) were assayed polarographically at 25°C. Coenzyme A was monitored with a dropping mercury electrode at a potential of –0.2 V relative to a saturated calomel anode with a Radiometer P04 recording polarograph [12, 13]. Citrate synthase was assayed in 0.1 M Tris–HCl, pH 8, with 0.2 mM oxaloacetate; the experiments on ATP inhibition were done in the presence of 0.05 mM acetyl-CoA. Reactions were initiated by the addition of enzyme.

Succinate thiokinase was measured in both directions. For the reaction succinyl-CoA to succinate, the assays were done in 0.1 M phosphate, pH 8, 10 mM Mg^{2+}, 0.5 mM ADP and succinyl-CoA as indicated. In the reverse direction, succinate to succinyl-CoA, assays were done in 0.1 M Tris–HCl, pH 8, 10 mM Mg^{2+}, 2 mM succinate, 0.05 mM CoA and ATP as indicated. All reactions were initiated by the addition of enzyme.

Measurements of citrate synthase and succinate thiokinase activities were made by using 20 μl of the toluenized cell suspension or 2μl of the cell-free extract.

It should be noted that these polarographic assays are particularly appropriate in the present studies as they are completely unaffected by the turbidity produced by the toluenized cells.

3. Results and discussion

We first examined the effect of ATP on the activity of citrate synthase in the cell-free extract of yeast. The results are shown in fig. 1 and clearly illustrate an inhibitory action of ATP essentially identical with that reported by Hathaway and Atkinson [2]. However, when similar measurements were made using the toluenized yeast cells no inhibition whatsoever of the citrate synthase activity was observed, even at an ATP concentration of 5 mM (fig. 1). It should be noted that this absence of ATP inhibition cannot be the result of treatment with toluene as the cell-free extract was also made from cells which had first been toluenized. Since the ATP inhibition is competitive with respect to acetyl-CoA [14] we thought it possible that the apparent absence of response to ATP might result from a lower K_m for acetyl-CoA exhibited by the enzyme *in situ* compared with that in extracts. When, however, the dependence of citrate synthase activity on acetyl-CoA concentration was determined, quite the reverse was found to be the case (fig. 2). Whereas the apparent K_m for acetyl-CoA was around 5 μM for the extracted enzyme (in agreement with the published value [14])

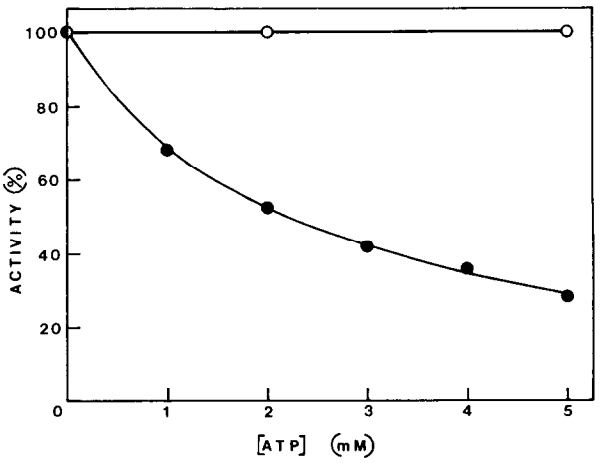

Fig. 1. Effect of ATP on citrate synthase activity. Assays were performed as described under Experimental using toluenized cells (○) or cell-free extract (●).

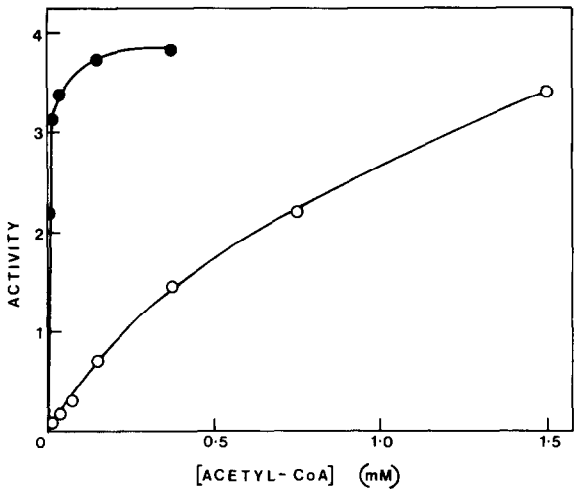

Fig. 2. Dependence of citrate synthase activity on acetyl-CoA concentration. Assays were performed as described under Experimental using toluenized cells (○) or cell-free extract (●). Activities are in arbitrary units.

a very much higher value in the region of 0.8 mM (over 100-fold increase) was exhibited by the enzyme *in situ*. This direction of change of the K_m for acetyl-CoA might have been expected to give rise to an even greater sensitivity of the *in situ* enzyme to ATP inhibition. The finding that citrate synthase in toluenized cells is not at all inhibited by ATP suggests that the conditions *in situ* maintain the enzyme in a form in which it cannot respond to ATP.

Consideration must, however, be given to the possibility that the differences observed between the behaviour of citrate synthase *in situ* and in extracts are due to residual permeability barriers in the toluenized yeast cells. Whereas the apparently simpler structure of bacterial cells may render permeabilization with toluene an acceptable means of gaining access to the intracellular enzymes, in the case of the more complex eukaryotic yeast cell the validity of measurements made on toluenized cells may need to be demonstrated before conclusions are drawn concerning the behaviour of enzymes *in situ*. This is particularly so in the case of those enzymes situated within sub-cellular organelles, e.g. the citric acid cycle enzymes inside the mitochondria. Thus, while treatment with toluene may break down the external permeability barriers of the cell it is conceivable that some internal barriers might withstand this treatment.

The observations reported above could therefore result from some impermeability of the toluenized yeast cells to acetyl-CoA and ATP. Severe restriction on the free diffusion of acetyl-CoA to the location of citrate synthase could account for the apparent high K_m and, similarly, inaccessibility to ATP would result in the absence of inhibition of enzymic activity.

We have attempted to examine this problem by making measurements of the activity of another citric acid cycle enzyme – succinate thiokinase. The intracellular location of the latter enzyme is likely to be very similar to that of citrate synthase and the accessibility of the two enzymes in toluenized cells to metabolites is likely to be the same. In addition, the succinate thiokinase reaction may be followed in either direction. It is thus possible to examine rate dependences on ATP in one direction and on succinyl-CoA in the other and we have assumed that accessibility to succinyl-CoA will be the same as that to acetyl-CoA in the permeabilized cell.

Allowing for the differences in concentration introduced in the course of their preparation, toluenized cells and the cell-free extract contained very similar levels of succinate thiokinase activity. The dependences on the concentrations of succinyl-CoA and ATP for permeabilized cells and extract are shown in figs. 3 and 4. These results indicate a fairly similar depen-

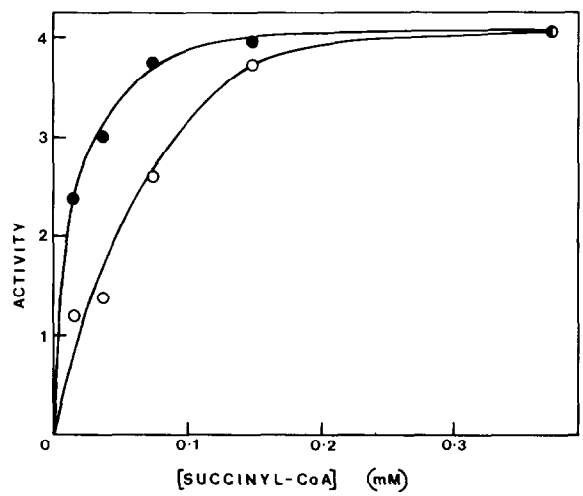

Fig. 3. Dependence of succinate thiokinase activity on succinyl-CoA concentration. Assays were performed as described under Experimental using toluenized cells (○) or cell-free extract (●). Activities are in arbitrary units.

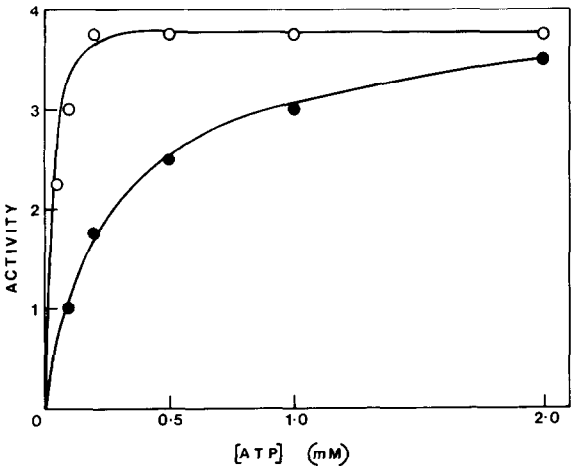

Fig. 4. Dependence of succinate thiokinase activity on ATP concentration. Assays were performed as described under Experimental using toluenized cells (○) or cell-free extract (●). Activities are in arbitrary units.

dence on succinyl-CoA for the enzyme in the two conditions. In the case of the dependence on ATP, the apparent K_m for ATP is noticeably lower for the enzyme *in situ*. Thus it would seem that toluenized yeast cells impose no restriction on the free movement of acyl-CoA or ATP to the site of the citric acid cycle enzymes.

4. Conclusion

We therefore conclude that the observed properties of yeast citrate synthase *in situ* are truly a reflection of the behaviour of the enzyme within the cell and that the different properties exhibited by the extracted enzyme arise out of the removal of the enzyme from this environment with the concomitant dilution and breakdown of molecular interactions. If indeed the true physiological behaviour of enzymes *in vivo* is more closely approximated by their behaviour *in situ* than by that exhibited in cell-free extracts, then our results would suggest that ATP inhibition of citrate synthase, at least in yeast, is not a physiologically significant regulatory mechanism.

Although, as mentioned above in the Introduction, citrate synthase isolated from various sources has been reported to be inhibited by ATP, low concentrations of Mg^{2+} have been found to reduce this inhibition [5, 6, 14, 15]. This finding has itself detracted from the appeal of ATP inhibition as a physiological control.

Garland [16] reported experiments on isolated rat liver mitochondria and suggested that the results were consistent with the control of citrate synthase by ATP and Krebs [17] has also presented evidence for the operation of this control in other tissues. However, studies by Olson and Williamson [18] led them to conclude that the regulation of citrate synthase in rat liver mitochondria cannot be correlated with the ATP level. In the case of yeast mitochondria the results of Garland et al. [19] suggested that the entry of acetyl-CoA into the citric acid cycle is not controlled by the ATP inhibition of citrate synthase.

More pertinent to the present work is the finding of Spivey and Srere, cited in [20], that increasing the concentration of citrate synthase (source undefined) reduced the inhibition by ATP. Srere had earlier drawn attention to the fact that intracellular concentrations of enzymes are very much greater than those normally employed in assays and that different enzymic behaviour might be associated with these different concentrations [21] and had posed the possibility that citrate synthase at its normal mitochondrial concentration might be unaffected by ATP [22].

The status of the control of citrate synthase by ATP is clearly unsettled. The present work, however, provides direct enzymological evidence for the insensitivity of yeast citrate synthase *in situ* to ATP and together with the findings of other investigators suggests the possibility that citrate synthase in some other types of cells may similarly be unaffected by ATP.

It is worth noting that the regulation of citrate synthase isolated from Gram-negative bacteria by NADH and AMP [23, 24] has been found to occur in the same manner *in situ* by examination of toluenized bacterial cells [9, 25].

Finally, the very high K_m for acetyl-CoA (approx. 0.8 mM) observed in the present study for yeast citrate synthase *in situ* might suggest that the availability of acetyl-CoA is a regulatory factor or that some unknown control mechanism operating *in vivo* can lower this parameter.

Our results emphasize the complexities of enzyme regulation and underline the fact that despite the numerous regulatory phenomena which have been

demonstrated *in vitro*, extrapolation to the *in vivo* situation is by no means straightforward. Indeed, in the absence of information concerning the modifying effects of the cellular environment on enzymic behaviour such extrapolation may be erroneous.

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