Succinate thiokinase in pigeon breast muscle mitochondria

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Succinate thiokinase has been purified from pigeon breast muscle. It has been confirmed that the enzyme is entirely specific for ATP, and the K_m is very high (~0.8 mM). Activity in mitochondrial sonicates is low enough for it to be doubtful whether the enzyme can support citric acid cycle flux in the tissue. The enzyme appears to have an M_r of 80000–100000, and to have two unequal subunits. As determined by SDS gel electrophoresis one subunit certainly has an M_r of 40000.

Succinate thiokinase *ATP specificity* *(Pigeon breast muscle)* *Mitochondria* *Michaelis constant*

1. INTRODUCTION

Previous work from this laboratory has shown that ATP-dependent succinate thiokinase (STK) enzymes, originally thought to be restricted to plants and bacteria [1], are also found widely but inconsistently in animal tissues [2]. In particular, the mitochondria of pigeon breast muscle, a tissue of very high oxidative capacity, have an ATP-dependent STK, and no trace of activity with GTP could be found [3]. This paper is a preliminary report on a partial purification of STK from this source, which has allowed the nucleotide specificity to be confirmed by two different assay methods, and an examination of some kinetic properties. The total activity of the enzyme in the starting material was low, and the apparent activity did not increase markedly at any stage during purification. If this had happened, it might have suggested that an endogenous inhibitor (functional or artifactual) had been removed. The low activity of the enzyme, in relation to the high oxidative capacity of pigeon breast muscle, has prompted us to consider the way in which the citric acid cycle can operate in pigeon breast muscle mitochondria.

Dedication: I have known Prakash Datta well for over 30 years. We began collaboration on a little book on biochemistry for medical students in 1952. In those days, and indeed for much longer, various editions of this book were known colloquially as 'the Aids'; it is a mark of how much times have changed that it would now be tactless, at least, to suggest that Prakash and Jim Ottaway were responsible for 'the AIDS'. It was therefore possible for me to see, at close range, the part that Prakash played both in the establishment of FEBS and FEBS Letters. What struck me most, apart from his executive efficiency and financial acumen, was the concern that he displayed for the needs of others, particularly scientific colleagues from countries outside the UK who might have, or be expected to have, financial difficulties, but also in ensuring that young scientists were also enabled to take as large a part as possible in FEBS activities. I experienced this at first hand when he helped me to organize one of the first FEBS Summer Schools in 1968, and I know that he was doing the same work, unobtrusive and behind the scenes, during the 14th FEBS Meeting in Edinburgh in 1981. It is, in my view, in no small measure due to Prakash that FEBS has become genuinely a continent-wide organization, and I think it is entirely appropriate that all papers in this issue of FEBS Letters should be dedicated to him. I wish him a long and happy retirement

2. MATERIALS AND METHODS

The enzyme was prepared from mitochondria of domesticated (racing) pigeons of various strains. About 80 g muscle from one or more pigeons was homogenized in a buffered mannitol medium [3], using a Waring blender operated at maximum

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power for 2 30-s bursts. Cell debris was spun down at $1000 \times g$ for 10 min, and the mitochondria were spun down at $15\,000 \times g$ for 10 min, and washed once with mannitol medium. This procedure obviated any possibility of contamination with nucleoside-diphosphate kinase [3]. Lipid material was removed by wiping the centrifuge bottles carefully with tissue.

Subsequent stages of purification were based on the procedure described by Hansford [5]. However, preliminary work confirmed that the enzyme is both somewhat more active and certainly more stable in Tris than in phosphate buffers [4,6]. Therefore, a Tris-succinate buffer ('preparation buffer'), consisting of Tris-succinate, 50 mM in succinate, pH 7.4, and containing 2 mM mercaptoethanol, was used. The mitochondria were resuspended in this buffer, and sonicated, in batches, in an MSE 60 W sonicator for 3 periods of 20 s, with pauses of 1 min between periods. The suspension was kept in ice during the sonication. The sonicate was centrifuged at $140\,000 \times g$ for 40 min at 2°C, and the resulting supernatant was used for further purification of the enzyme by ammonium sulphate fractionation, retaining the fraction precipitating between 260 and 450 g ammonium sulphate per l of original supernatant. The ammonium sulphate was added slowly to the solution held at 5°C, and the pH was adjusted to remain not lower than 7.0.

The protein was resuspended in preparation buffer, and dialyzed for 3 h against 2 changes of buffer. The concentration of protein was adjusted to about 30 mg/ml, and ~8 ml (250 mg protein) was passed rapidly (40 ml/h) down a column of Ultrogel AcA 44 (LKB), M_r size 12 000–130 000, which had previously been equilibrated in the preparation buffer. The column dimensions were 3.5 cm diameter and 90 cm in length. Protein elution was followed by measuring absorbance at 280 nm, checked by protein estimation by Lowry's method.

Enzyme activity was assayed by measuring changes in absorbance at 235 nm, using a Perkin-Elmer 5000 S spectrophotometer, checked for linearity up to an absorbance of 2.9. 1 mM ATP was used in place of GTP. Assays were run at 25°C.

3. RESULTS

3.1. *Enzyme purification*

All the activity appeared in a single peak with highest specific activity at tube 11 after protein breakthrough. Table 1 shows that the overall recovery of the enzyme was about 70%, and that there had been a 45-fold purification. Although the specific activity of the most highly purified fraction was low by comparison with those quoted elsewhere [1,5], SDS-polyacrylamide gels showed only a few minor contaminants, and the major fraction of the column eluate appeared to be about 80% pure, in terms of protein. As can be seen from table 1, there was very little loss of activity during the purification. It is possible that there was major inactivation during the preparation of the mitochondria or during sonication, but the figures quoted here for the sonicate supernatants agree well in those obtained for the activity of STK in pigeon breast muscle mitochondria prepared on a

Table 1

Purification of pigeon breast muscle STK

	Total activity (μmol/mg)	Total protein (mg)	Specific acitivity (μmol/min per mg)
Sonicate supernatant	238	7000	0.034
Ammonium sulphate precipitate	180	1000	0.18
Pooled column eluate	173	120	1.5

small scale and rapidly fragmented either by sonication [3] or by osmotic shock (M.L. Hamilton, unpublished).

There was a pronounced shoulder of activity at the leading (higher M_r) shoulder of the chromatographic peak, suggesting that the enzyme was not homogeneous. This was confirmed by SDS-polyacrylamide gel electrophoresis (SDS-PAGE), both of the ammonium sulphate fraction, and of the column eluate, and confirms earlier observations (reviewed in [1]). No attempt was made rigorously to estimate the M_r of the native enzyme, but as it was not completely excluded from the column, it must have been $< 130\,000$. The M_r of the subunits found on SDS-PAGE was 40 000 and 48 000 (fig. 1); this suggests that the native enzyme might be a dimer of M_r 80 000–100 000. This is of the same order of magnitude as that reported for mammalian STK [7].

3.2. *Specificity and kinetic studies*

As reported previously, the enzyme was absolutely specific for ATP. As it was prepared from mitochondria, contamination by nucleoside-diphosphate kinase (NDK) could be excluded [3]. The specificity was confirmed by polarographic measurement of activity in the reverse (ADP-utilizing) direction (D. Weitzman, personal communication). The only other report of STK preparation from pigeon breast muscle [6] makes it clear that GTP was the substrate. The properties of the enzyme described in [6] were in many other ways similar to those of our own preparation (N.B. The earlier preparation is wrongly attributed to pigeon liver in [1].) The discrepancy cannot be explained, but it may be significant that the earlier workers used minced whole breast muscle as starting material. It is possible that one of the two bands that they observed on their electrophoresis gels contained some NDK.

The assay method used is not suitable for estimating the K_m of the enzyme for CoA, but measurements were made at 3 different concentrations of this substrate (in the presence of 1 mM ATP), and the data were compatible with the value for the K_m for CoA reported for other STK species, namely approx. 0.02 mM [4]. The K_m for ATP, however, was much higher than for any GTP-dependent STK for which data have been reported [1,4]. It was about 0.8 mM (fig. 2). Hansford [5] also reported that the K_m for ATP of the ATP-dependent STK isolated from blowfly

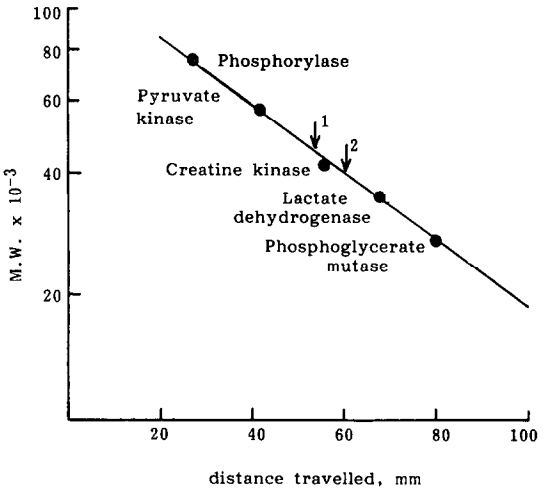

Fig. 1. SDS-PAGE of STK subunits. The marker proteins were enzymes from chicken breast muscle. Subunit (2) was predominant in the fraction of highest specific activity from the Ultrogel exclusion column. See text.

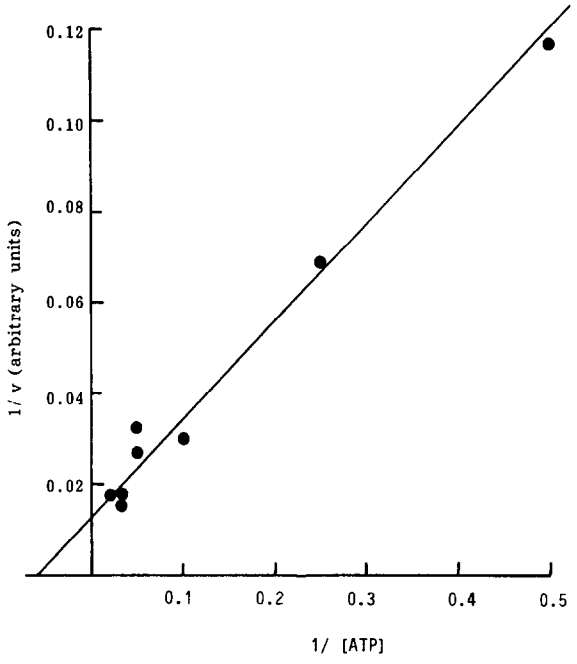

Fig. 2. Double-reciprocal plot of STK activity at varying ATP concentrations.

flight muscle was higher than that for GTP-dependent enzymes (~0.3 mM). His measurements were made in 40 mM phosphate buffer and it is possible that somewhat higher apparent K_m might be found in a phosphate-free medium.

Calculation shows that at 10 mM [total Mg^{2+}], the conditions used in all our assays, ATP would be almost exclusively present as the species Mg-ATP^{2-}. The linearity of the double-reciprocal plot shown in fig. 2 suggests that this species is the true substrate. This must certainly be true for the GTP-dependent enzymes whose kinetics have so far been studied. Whether free ATP is a competitive inhibitor (cf. [5]) is an interesting question which deserves further study.

Table 2

(A) Intact mitochondria (state 3) – rate of substrate oxidation (ngatom O/min per mg protein). Substrate:	
ascorbic acid + TMPD	970
succinate	255
pyruvate/malate	182
glutamate/malate	215
α-oxoglutarate	243
(B) Enzyme activity in sonicates (nmol substrate used/min per mg protein)	
malate dehydrogenase	1210
succinate thiokinase	34

All values are the mean of two observations. For further data see [3]

4. CONCLUSIONS

It is of great significance that the affinity for ATP of an ATP-dependent enzyme, located in the mitochondrial matrix, is much less than the affinity of similar GTP-dependent enzymes for GTP (using K_m values as a rough measure of substrate affinity). This is because the concentration of ATP in the matrix is at least 20-fold higher than that of GTP [8]. Equilibrium enzymes work in any case far below their optimal activity [9], effectively because of product inhibition. One may easily calculate that if the K_m of the pigeon breast muscle enzyme for ATP were to be as low as the values reported for the K_m for GTP of the GTP-dependent enzyme, STK in this tissue would be almost completely inactive. The 40-fold difference in K_m values reported here strengthens the view, which has been held consistently in this laboratory, that except for enzymes held in membranes or other non-aqueous environments, enzymes operate in vivo much as they do in vitro. This means that computer simulation of enzyme systems based on in vitro data is a worthwhile practice, and, as a corollary, that it is still worthwhile to obtain accurate kinetic data about individual enzymes.

We remain, however, unconvinced that STK plays a major role in citric acid cycle metabolism in pigeon breast muscle, or in other active vertebrate muscles, because of the low level of activity of the enzyme reported in the literature (for review see [10]). The situation may be very different in liver [8], but as we have shown elsewhere [11], the maximum flux rate which it is possible to obtain in simulation experiments does not match the experimentally determined flux through the cycle.

Table 2 shows that there is a major discrepancy between the observable rates of substrate oxidation in intact mitochondria, and the measurable activity of STK in sonicates. This discrepancy does not exist for malate dehydrogenase, for example. In earlier publications [2,3,10,11] we have explored the possible role of acetoacetate activation as a possible means of utilizing succinyl-CoA and so bypassing STK, but ketone body metabolism does not seem to be very high in the pigeon, and this explanation does not at present seem to be adequate to explain the results reported in table 2.

STK is important not only for the flux of succinate in the citric acid cycle, but also as a major source of the free CoA required by oxoglutarate dehydrogenase. More than half of the free CoA in the mitochondrial matrix can be shown by simulation studies to be sequestered on citrate synthase (J.H. Ottaway, unpublished). It is very difficult to see how a fast response of oxoglutarate dehydrogenase, which must accompany a sudden increase of physiological activity in heart or avian flight muscle [11], can be provided for by such a sluggish enzyme as STK. The existence of a Ca^{2+}-dependent succinyl-CoA hydrolase would provide a satisfactory explanation of an important, but still unsolved, problem in the regulation of mitochondrial metabolism.

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