# Occurrence of two distinct succinate thiokinases in animal tissues

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Although succinate thiokinase from mammalian sources has hitherto been described as showing substrate specificity for guanine nucleotide, a range of mammalian tissues has here been found to display succinate thiokinase activity with both guanine and adenine nucleotides as substrates. Evidence is presented for the existence of two distinct succinate thiokinases and this is confirmed by their separation by affinity chromatography. Each enzyme is specific for one nucleotide and is inhibited by the non-substrate nucleotide. The physiological roles of the two enzymes is yet to be established.

*Adenine nucleotide* *Guanine nucleotide* *Nucleotide specificity* *Succinate thiokinase* *Tissue enzyme variation*

## 1. INTRODUCTION

Succinate thiokinase (succinyl-CoA synthetase) (STK) catalyses the following reversible reaction as part of the citric acid cycle:

$$NDP + P_i + \text{succinyl-CoA} \rightleftharpoons NTP + \text{succinate} + \text{CoA-SH}$$

where NDP and NTP represent nucleoside diphosphate and triphosphate. The STKs which have been most fully studied are those from pig heart and *Escherichia coli* [1,2]. The pig STK was found to function with GDP/GTP as substrates, while the *E. coli* enzyme appeared to be specific for ADP/ATP. These findings led to the established view that animal STKs utilize guanine nucleotides whereas bacterial (and plant) STKs operate with adenine nucleotides, a situation generally presented in textbooks of biochemistry. Some years ago, however, an adenine nucleotide-linked enzyme (A-STK) was reported to occur in blowfly flight muscle [3] and, more recently, A-STK activity has been found in a wider range of animals [4,5], though not in mammals. In some non-mammalian tissues, both A-STK and G-STK activities were detected [4,5] but whether these activities result from a single non-specific enzyme or from the presence of two specific STKs was not resolved.

We have previously reported considerable variation in the nucleotide specificities of a range of bacterial STKs and have shown that some of the bacterial enzymes function as well, or even preferentially, with GDP rather than ADP [6]. In the light of this diversity and the apparent breakdown of the earlier view concerning animal and bacterial STKs it seemed worthwhile to examine carefully the STKs of mammalian tissues for the possible occurrence of both types of STK activity. This communication reports, for the first time, the presence of both A-STK and G-STK activities in mammals and presents clear evidence for their location on two distinct enzyme proteins.

## 2. EXPERIMENTAL

### 2.1. *Preparation of tissue extracts*

Fresh beef, pig and sheep tissues were obtained from a local slaughterhouse, immediately cooled on ice and processed in the laboratory within 1 h of slaughter. Rat tissues were taken from freshly

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killed male Wistar rats. Tissues were manually diced into ice-cold 0.1 M Na/K phosphate buffer (pH 8.0) containing 1 mM EDTA, and homogenized with an Ultra-Turrax tissue homogenizer (5 × 15 s bursts interspersed with cooling). After centrifugation (30000 × *g* for 30 min at 4°C) the supernatant solutions were used without further treatment.

### 2.2. *Preparation of beef heart mitochondrial extract*

Mitochondria were prepared from a whole beef heart as in [7] with the slight modification that they were washed twice in 50 mM Tris-HCl (pH 7.5), 0.25 M sucrose, 1 mM EDTA. The washed mitochondria were resuspended in 20 mM Tris-HCl (pH 7.5), 2 mM $MgCl_2$, and disrupted by ultrasonication (MSE 100 W sonicator operated at 40 W for 6 × 15 s with cooling). After centrifugation (30000 × *g* for 30 min at 4°C) the supernatant solution was used in the experiments reported.

### 2.3. *Assay of succinate thiokinase*

STK was assayed polarographically [8,9] as described in [6] in the presence of 0.5 mM ADP or GDP.

### 2.4. *Thermal inactivation*

2.7 ml of 0.1 M Na/K phosphate (pH 8.0), 1 mM EDTA were equilibrated at 48°C. At zero time, 0.3 ml tissue extract was added and aliquots (0.3 ml) were removed at various time intervals and assayed for both A-STK and G-STK activities.

### 2.5. *Affinity chromatography*

Affinity chromatography was carried out on derivatized GDP immobilized on Sepharose [10]. A column (1.5 × 15 cm) of this affinity material was equilibrated at 4°C with 20 mM Tris-HCl (pH 7.5), 2 mM $MgCl_2$ and 0.5 ml of the beef heart mitochondrial extract (~12 mg protein) was applied. Elution was continued with the equilibration buffer, 1-ml fractions being collected. When the protein content of the eluted fractions had fallen to zero, the elution buffer was changed to include 1 mM GDP. Removal of GDP from the subsequent fractions apparently containing STK was achieved by pooling and gel filtration on a prepacked Sephadex G-25 column (Pharmacia, PD-10).

## 3. RESULTS AND DISCUSSION

As stated in section 1, all previous studies on mammalian STK have emphasised the specificity of the enzyme for guanine nucleotide substrates (GDP or GTP, depending on the direction of assay of the reaction) and no observation of activity with ADP/ATP has hitherto been reported.

Our first significant finding then is that all the mammalian tissue extracts examined showed STK activity with both ADP and GDP. This was the case with bone marrow and heart from beef, brain, heart, kidney, liver and retina from pig, brain, heart, kidney, liver and skeletal (leg) muscle from rat, and brain and liver from sheep. A single sample of human leg muscle (following hospital amputation) was also found to display activity with both nucleotides. Measurements of STK activity were greatly facilitated by our use of the polarographic method of enzyme assay. We have previously commented [6,11] on the superiority of this assay in comparison with the discontinuous colorimetric hydroxamate method [12] or the spectrophotometric method at 235 nm [13] used by other investigators. Our discovery of STK in cyanobacteria [11], despite its earlier reported absence, owed much to the use of the polarographic assay and the present observations of both A-STK and G-STK activities in mammalian tissues may also have eluded other investigators employing less favourable detection procedures.

The next significant finding was that the ratio of the A-STK and G-STK activities varies between tissues (table 1). A spectrum of ratios was found,

Table 1

Activities of G-STK and A-STK in extracts of various rat tissues

| Tissue | Activity (nmol/min per mg protein) | | Ratio G-STK/A-STK |
|---|---|---|---|
| | G-STK | A-STK | |
| Kidney | 6.87 | 1.96 | 3.5 |
| Liver | 4.54 | 1.5 | 3.0 |
| Heart | 3.58 | 2.5 | 1.4 |
| Brain | 0.95 | 1.51 | 0.6 |
| Skeletal muscle | 0.42 | 1.06 | 0.4 |

the G-STK being the predominant enzyme in kidney and liver, more nearly matching the A-STK in heart, and being exceeded by the A-STK in brain and skeletal muscle. It is difficult to reconcile this approx. 10-fold change in the G-STK/A-STK ratio with the presence of a single STK enzyme of loose specificity; one would have to postulate that the 'looseness' of the specificity itself varied between tissues. A simpler explanation is that there are two, nucleotide-specific, STKs whose relative proportions differ between tissues. Ottaway and coworkers [4,5], who first reported A-STK and G-STK in a number of non-mammalian animal tissues, also observed a divergence in the ratio and commented that this may be an indication of the presence of two enzymes, but no further experimental evidence was presented.

The ability of a guanine nucleotide-specific STK to show activity with ADP could result from the additional presence of nucleoside-diphosphate kinase (NDPK) activity and traces of GTP (see [2]). However, the apparent activity of A-STK could never exceed that of G-STK. Thus the fact that A-STK was the major activity in brain and muscle excludes the possibility that A-STK activity results from the action of NDPK. Furthermore, the inclusion of EDTA in the isolation and washing media used in the preparation of beef heart mitochondria has the effect of removing NDPK [5].

The heart mitochondrial preparation also showed the presence of A-STK and G-STK, indicating that both activities are located within the mitochondria. The mammalian G-STK is known

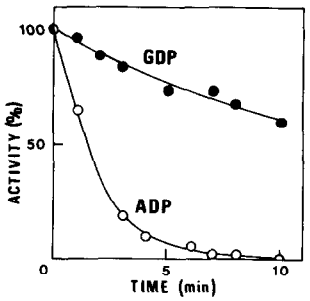


Fig.1. Thermal inactivation of rat heart succinate thiokinases at 48°C. Experimental conditions are described in the text.

to be a dimer of $M_r$ ~70000 [2]. We therefore compared the molecular sizes of the G-STK and A-STK identified in mammalian tissues by means of gel filtration on Sephadex G-200 as in [14]. The two enzyme activities from all the rat tissues listed in table 1 co-eluted on gel filtration, indicating no detectable difference in molecular size between the A-STK and G-STK activities.

One way to test for the presence of two distinct enzymes, as opposed to a single STK with broad specificity, is to compare the activities measured in the presence of ADP and GDP singly with that measured in their joint presence. A single enzyme saturated with the 'preferred' nucleotide would not show increased activity on addition of the second nucleotide. On the other hand, if two enzymes are present, saturation of one of them with its specific nucleotide should still permit the other enzyme to display activity on the addition of the other nucleotide; hence some additivity of activities

Table 2

Specificity of the separated succinate thiokinases and inhibition by the non-substrate nucleotide

| Enzyme | Activity (nmol/min per ml) | | | Inhibition (%) |
|---|---|---|---|---|
| | 0.5 mM ADP | 0.5 mM GDP | 0.5 mM ADP + 0.5 mM GDP | |
| A-STK | 38.0 | 0. | 24.5 | 35 |
| G-STK | 0 | 99.5 | 65.5 | 34 |

The two STKs from bovine heart mitochondria were separated by affinity chromatography as described in the text

should be observed. In [4], additivity of the activities of STK with GTP and ATP was used as a criterion for the genuine utilization of both nucleotides (rather than an artefactual display of A-STK activity due to nucleoside diphosphate kinase), though not for the existence of distinct STKs. In the present work, the activities of STK in several tissue extracts (beef heart, pig brain, rat heart) measured in the joint presence of ADP + GDP (both at 0.5 mM, i.e. above saturation) were greater than either of the activities measured in the presence of a single nucleotide. The additivity observed was not strictly the sum of the two activities, but partial additivity (between 50 and 100%) was clearly seen and thus offers further evidence for the existence of two STKs. A possible explanation for the partial additivity is that although each STK is specific for one nucleotide as substrate, the other nucleotide may act as an inhibitor; this point is considered further below.

Additional evidence for the two STKs has been obtained from thermal inactivation studies. Rat heart extract showed the A-STK to be more rapidly inactivated at 48°C than the G-STK (fig.1), supporting the non-identity of the A-STK and G-STK proteins. Similar results were obtained with pig brain.

Finally, conclusive evidence for distinct enzymes was obtained by separating the two activities by affinity chromatography. When the beef heart mitochondrial extract was applied to a column of dialdehyde-GDP-Sepharose (see section 2.5) only the A-STK activity could be detected in the fractions eluted when the column was washed with the equilibration buffer. However, when this buffer solution also contained 1 mM GDP, the G-STK activity was sharply eluted. Neither enzyme showed any activity whatsoever with the other nucleotide. These findings clearly demonstrate the existence of two distinct STKs – one specific for adenine and the other for guanine nucleotide. Table 2 shows that, in each case, the non-substrate nucleotide acts as an inhibitor of the 'other' activity; these inhibitions therefore support the explanation offered above for the observation of only partial additivity when activity measurements were made with crude extracts in the joint presence of both nucleotides.

Our results thus demonstrate that, despite textbook assertions, mammalian cells do possess the enzymic facility to produce ATP directly through citric acid cycle activity. The question of why mitochondria contain two STKs remains to be answered. It is conceivable that they differ in their location or fine level of organisation within the mitochondrion and they may play distinct metabolic roles. It has been suggested [4] that there may be a connection between G-STK activity and ketone body metabolism. Now that we have demonstrated the presence of both G-STK and A-STK in mammals, with tissue-dependent variation, investigations are in progress to explore their particular physiological functions.

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