# AN ADENINE NUCLEOTIDE-LINKED SUCCINIC THIOKINASE OF ANIMAL ORIGIN

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## 1. Introduction

The complete oxidation of pyruvate by mitochondria isolated from blowfly flight muscle is markedly more rapid in the presence of high intra-mitochondrial concentrations of ADP and phosphate [1]. During a study of tricarboxylate cycle enzymes which might be responsible for this effect, it has emerged that succinic thiokinase activity from fly flight muscle differs from that described from any other animal source in reacting with adenine, and not guanine, nucleotides.

## 2. Materials and methods

The enzyme was prepared from 20 g of thoraces of *Calliphora erythrocephala*, isolated after freezing the flies at the temperature of solid $CO_2$/acetone. Thoraces were disintegrated in 200 ml of 50 mM potassium phosphate pH 7.2, 2 mM mercaptoethanol, 1 mM EDTA ("preparation buffer") using a Waring blendor at maximum power for 2 min. Debris was removed by filtering through cheesecloth and the resulting homogenate was sonicated in 4 batches for 3 periods of 20 sec using an MSE 60 W sonicator, to fragment any intact mitochondria. The suspension was then centrifuged at 140,000 g for 40 min and the resulting supernatant used for further purification. Succinic thiokinase was precipitated between 286 and 445 *g* of ammonium sulphate per ℓ of original supernatant. The protein was re-dissolved in 5 ml of preparation buffer and subjected to molecular sieving on an Agarose A5m column (1.2 cm radius × 50 cm). Hydroxylapatite gel (1 g dry weight) was added to the pooled fractions in a total volume of 70 ml of 50 mM phosphate pH 7.4. The gel was sedimented, washed with 20 ml of 0.2 M phosphate pH 7.4, sedimented and then washed twice with 3 ml portions of 0.4 M phosphate pH 7.4. This eluted the enzyme. Protein was determined according to Cha [2].

Enzyme activity was assayed by monitoring changes in $A_{235}$, according to Cha [2]. This involved very high total absorbance but was possible using a Hilger-Gilford spectrophotometer, checked as being linear up to an absorbance of 2.9. At the high nucleotide concentrations used in the backwards reaction this assay was not feasible and was replaced by measurement of ADP formation using pyruvate kinase and lactate dehydrogenase [2]. All experiments were at 25°.

Succinyl coenzyme A was prepared according to Simon and Shemin [3]. Free coenzyme A was removed by passing the product down a column of Agarose-*p*-amino phenyl mercuric acetate, (Miles-Yeda), followed by a column of Sephadex G-10, on a suggestion by Dr. P.J. Winterburn. The resulting solution contained no coenzyme A, as estimated by the method of Ellman [4] and was stored frozen at pH 1.5, and neutralised within a few hours of use. Succinyl coenzyme A was assayed by allowing the enzymic reaction to reach equilibrium at high phosphate and ADP concentrations and assuming a millimolar extinction coefficient ($A_{235}$) of 4 [5].

## 3. Results

Table 1 shows that a three-stage partial purification gave a 60-fold increase in specific activity. In fact, 0.25 mM ATP gives only 50% of $V_{max}$ and so these values of specific activity may be doubled,

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Table 1
Purification of succinic thiokinase from blowfly flight muscle.

| Stage of preparation | Total activity (μmoles/min) | Total protein (mg) | Specific activity (μmoles/min/mg) |
|---|---|---|---|
| Original (144,000 *g*) supernatant | 299 | 1760 | 0.17 |
| Ammonium sulphate precipitate | 267 | 339 | 0.76 |
| Pooled fractions from A5m column | 148 | 51 | 2.9 |
| Eluate from hydroxyl-apatite | 57 | 5.7 | 10 |

Activity was measured in 50 mM N-tris-(hydroxymethyl)-methyl-2-amino ethanesulphonic acid (TES), 72 mM KCl, pH 7.4 containing 50 mM potassium succinate pH 7.2, 10 mM $MgCl_2$, 60 μM coenzyme A and 0.25 mM ATP.

giving a result of 20 Units/mg, which is close to values for the pure enzyme from other tissues [2, 6].

GDP, UDP, CDP, IDP and AMP all gave negligible activity in the forward reaction, and GTP, UTP, CTP and ITP gave negligible activity in the reverse direction – in each case less than 2% of the rate with adenine nucleotide at the same concentration. The thioester, 235 nm, assay was used.

The apparent $K_m$ values for adenine nucleotides were found to be high, relative to the values obtained by Cha and Parks [7] for the pig heart enzyme and guanine nucleotides. Fig. 1 shows the dependence upon ATP concentration of the reverse reaction and indicates an apparent $K_m$ of 0.29 mM ATP, at saturating concentrations of cosubstrates. Fig. 2 presents data for the dependence of the rate of the forward reaction upon ADP concentration and suggests an apparent $K_m$ of 0.12 mM at saturating phosphate concentrations. It was unfortunately not possible to use a saturating level of the other cosubstrate, succinyl coenzyme A. Similar kinetic plots (not shown) gave the further data compiled in table 2. It is seen that the apparent $K_m$ for ADP increases with pH, whilst the

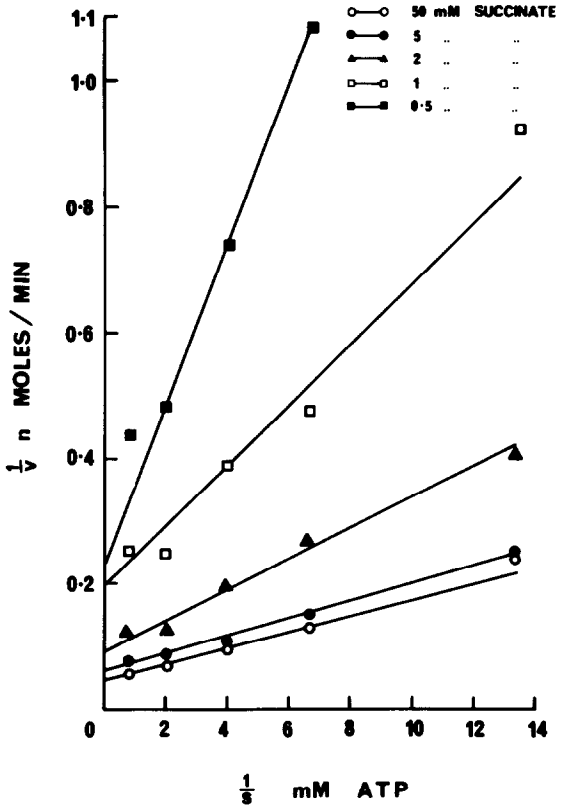


Fig. 1. A double reciprocal plot showing the effect of ATP concentration on initial velocity at different concentrations of succinate. Each cuvette contained 40 mM potassium phosphate pH 7.2, 10 mM $MgCl_2$, 0.06 mM coenzyme A and the concentrations of succinate and ATP shown. Apparent $K_m$ values for ATP at 50, 5, 2, 1 and 0.5 mM succinate were 0.29, 0.24, 0.28, 0.25 and 0.59 mM, respectively.

apparent $K_m$ for phosphate decreases. This may reflect a requirement for the species $ADP^{2-}$ and $phosphate^{2-}$ respectively.

## 4. Discussion

Succinic thiokinase from fly flight muscle clearly differs from the enzyme described from other animal sources [2, 5, 7] in reacting with adenine and not guanine nucleotides. To that extent it resembles the enzyme from plant tissues, EC 6.2.1.5 [8, 9]. It would be of interest to investigate the nucleotide dependence of the enzyme from fly fat body, to determine

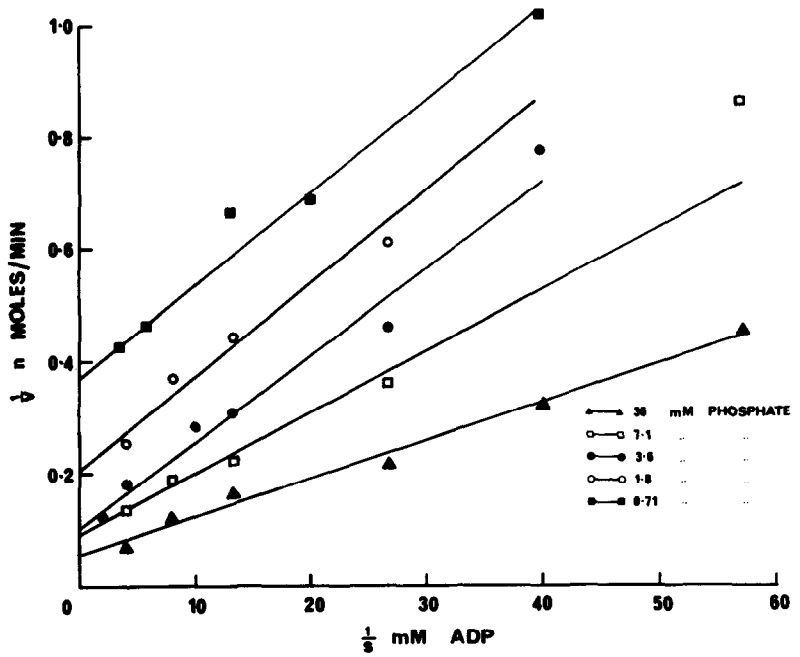


Fig. 2. A double reciprocal plot showing the effect of ADP concentration on initial velocity at different concentrations of phosphate. Each cuvette contained volumes of 50 mM phosphate pH 7.2 and 50 mM TES, 55 mM KCl pH 7.2 buffers to give the final phosphate concentration indicated, 10 mM $MgCl_2$ and 0.03 mM succinyl coenzyme A. Apparent $K_m$ values for ADP at 36, 7.1, 3.6, 1.8 and 0.71 mM phosphate were 0.12, 0.12, 0.15, 0.08 and 0.05 mM, respectively.

Table 2
Kinetic constants of forward and reverse reactions.

| Variable substrate | Concentration of other substrates (mM) | | | Other conditions | Apparent $K_m$ of variable substrate (mM) |
|---|---|---|---|---|---|
| | Succinyl-coenzyme A | ADP | Phosphate | | |
| (A) Forward reaction | | | | | |
| *Succinyl coenzyme A | – | 0.40 | 40 | – | 0.024 |
| ADP | 0.075 | – | 40 | pH 6.8 | 0.081 |
| | | | | pH 7.2 | 0.13 |
| | | | | pH 7.4 | 0.29 |
| Phosphate | 0.033 | 0.40 | – | pH 6.8 | 16 |
| | | | | pH 7.1 | 3.4 |
| | | | | pH 7.4 | 1.9 |
| (B) Reverse reaction | Coenzyme A | ATP | Succinate | | |
| Coenzyme A | – | 2.4 | 25 | – | 0.010 |
| Succinate | 0.060 | 2.5 | – | 1 mM phosphate | 2.2 |
| | | | | 40 mM phosphate | 3.0 |
| ATP | 0.060 | – | 50 | 40 mM phosphate | 0.29 |

The buffer used in these experiments was potassium phosphate where indicated, otherwise 50 mM TES/KCl of the same pH and ionic strength. The pH was 7.2 throughout, except where indicated. The pyruvate kinase/lactate dehydrogenase assay was used for all experiments in section B.

* The succinyl coenzyme A contained 11% coenzyme A due to hydrolysis after preparation. $K_i$ for coenzyme A = 0.015 mM.

whether or not adenine nucleotide specificity is an adaptation to the simplified metabolism of flight muscle.

It is difficult to assess from these results whether the enzyme could have a role in controlling the flux through the tricarboxylate cycle, as it was not possible to use intra-mitochondrial levels of adenine nucleotides in the 235 nm assay, and an assay based on the production of ATP in the forward reaction was foiled by contaminating activities in the commercial glucose-6-phosphate dehydrogenase and hexokinase. Nevertheless, it seems a real possibility, as ATP inhibits ADP binding (competitive with a $K_i$ of 0.11 mM at pH 6.8, mixed kinetics at pH 7.4) and the mitochondrial content of ATP probably exceeds that of ADP at rest.

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