Citrate synthase in the rainbow trout heart: regulation by pH, temperature, and metabolite levels

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Crude homogenates of rainbow trout (*Oncorhynchus mykiss*) hearts have high citrate synthase activity (30 units per gram wet weight at 20°C), reflecting the aerobic nature of this organ. Citrate synthase was purified 70-fold by polyethylene glycol fractionation followed by ion-exchange separation on phosphocellulose and DEAE columns. At 20°C the partially purified enzyme displayed hyperbolic Michaelis–Menton-type substrate kinetics and an activity maximum at pH 8.5. The K_m values for oxaloacetic acid and acetylcoenzyme A were 1.65 and 3.91 μM, respectively. Competitive inhibition with respect to oxaloacetic acid was seen with citrate ($K_i = 8.80$ mM) and α-ketoglutarate ($K_i = 13.95$ mM). Noncompetitive inhibition by ATP ($K_i = 1.04$ mM) with respect to oxaloacetic acid was observed. ATP ($K_i = 0.382$ mM) and NADH ($K_i = 0.451$ mM) were competitive inhibitors for acetylcoenzyme A. Citrate synthase activity is both pH- and temperate-dependent. Indeed, the temperature dependence (0–25°C) is modified by pH. This is reflected by a decrease in the activation energy from 15.7 to 10.6 kcal/mol (65.63 to 44.30 kJ/mol) as the pH of the medium is increased from 7.05 to 7.80. These findings are discussed with respect to trout heart citrate synthase function *in vivo*.

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L'activité élevée de la citrate synthase (30 unités par gramme de masse fraîche à 20°C) dans des homogénats bruts de coeurs de Truite arc-en-ciel (*Oncorhynchus mykiss*) reflète la nature aérobique de cet organe. La citrate synthase a été purifiée (×70) par fractionnement au polyéthylène glycol suivi d'une séparation par échange d'ions sur de la phosphocellulose et des colonnes DEAE. À 20°C, la cinétique de l'enzyme partiellement purifiée suit une courbe hyperbolique de type Michaelis–Menton et l'activité est maximale à pH 8,5. Les valeurs de K_m ont été estimées à 1,65 dans le cas de l'acide oxaloacétique et à 3,91 μM dans le cas de l'acétyl coenzyme A. Une inhibition compétitive à l'égard de l'acide oxaloacétique a été constatée dans le cas du citrate ($K_i = 8,80$ mM) et de l'α-cétoglutarate ($K_i = 13,95$ mM); une inhibition non compétitive par l'ATP ($K_i = 1,04$ mM) a été observée. L'ATP ($K_i = 0,382$ mM) et le NADH ($K_i = 0,451$ mM) se sont avérés des inhibiteurs compétitifs pour l'acétyl coenzyme A. L'activité de la citrate synthase dépend à la fois de la température et du pH, puisque l'effet de la température (0–25°C) est modifié par le pH. Cette tendance a été mise en évidence par une diminution de l'énergie d'activation de 15,7 à 10,6 kcal/mol (65,63 à 44,30 kJ/mol) lorsque le pH du milieu augmente de 7,05 à 7,80. Ces résultats sont examinés en fonction du rôle de la citrate synthase dans le coeur de la truite *in vivo*.

[Traduit par la rédaction]

Introduction

Aerobic metabolism plays an integral role in the production of the energy required for work in the teleost heart (Farrell 1984; Turner and Driedzic 1980; Lanctin *et al.* 1980). During strenuous burst or sustained exercise, the dynamic changes observed in the cardiovascular system are complex and are supported by a 3- to 5-fold increase in cardiac output (e.g., Jones and Randall 1978). Recent experiments with rainbow trout heart *in situ* have demonstrated that myocardial power output is linearly and positively correlated with cardiac oxygen consumption (Graham and Farrell 1990) and, presumably, with tricarboxylic acid (TCA) cycle activity. Being the first step of the TCA cycle, the enzymatic formation of citrate from oxaloacetic acid (OAA) and acetylcoenzyme A (ACoA) represents an important potential regulatory locus for overall energy production in the heart. Indeed, citrate synthase (citrate oxaloacetate-lyase (CoA-acetylating), EC 4.1.3.7) is regarded as a key control enzyme of the TCA cycle (e.g., Srere 1975). Nevertheless, nothing is known of the regulatory properties of fish heart citrate synthase. To evaluate some factors that may influence the activity of this enzyme, we partially purified and determined some kinetic characteristics of rainbow trout (*Oncorhynchus mykiss*) heart citrate synthase *in vitro*.

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Materials and methods

Animals

The hearts used in this study were collected from rainbow trout immediately following harvest in May at the fish hatchery of La Reserve de la Petite Nation, Montebello, Quebec. The animals had been raised in outdoor freshwater pens (temperature ~15°C), were of both sexes, and ranged in size from 350 to 500 g. The fish were netted and quickly killed by electrocution prior to being prepared for commercial sale. At this time, the hearts were removed, blotted on tissue paper, frozen in liquid nitrogen, and transported to our laboratory in Ottawa, where they were stored at −80°C until use (<1 week).

Other tissues were used for obtaining comparative maximal activity data (Table 1). Livers were harvested at the same time and in the same manner as the hearts (see above). White muscle and gill tissues were collected in early June from rainbow trout held at the aquatic laboratories of the University of Ottawa. The animals were quickly netted from indoor tanks (freshwater, ~15°C) and killed by a blow to the head. The organs were dissected out and frozen in liquid nitrogen prior to transport and storage at Carleton University as described above.

Preparation and partial purification of trout heart citrate synthase

The whole-heart tissue (approximately 1 g) was homogenized (1:5 w/v) in ice-cold buffer (10 mM KPO_4, pH 7.5, with 0.01 mM phenylmethylsulfonylfluoride added immediately before use) by three 10-s bursts of an Ultra Turrax tissue homogenizer. The resulting homogenate was centrifuged at 27 000 × *g* in a Sorvall RC-5B ultracentrifuge thermostatted at 5°C. Analysis of the supernatant revealed that 10% or

less of the total citrate synthase activity remained associated with the protein pellet. This pellet was discarded and the supernatant of the centrifuged crude homogenate (approximately 4 mL) was treated with 12% polyethylene glycol (PEG). The PEG mixture was inverted gently at 5°C for 10 min, after which the mixture was centrifuged for 5 min at 17 000 rpm in an Eppendorf centrifuge. The resulting protein pellet was discarded and the supernatant was treated with 20% PEG. Following mixing and centrifugation as before, the final protein pellet was resuspended in 3.5 mL homogenization buffer.

The fractionated protein mixture was applied to a 1.5 × 2.5 cm phosphocellulose column (Sigma C2383, equilibrated with homogenization buffer) and eluted with a 0–2 M KCl gradient (Fig. 1A). One-millilitre fractions were collected and those containing 50% or more of the citrate synthase activity found in the maximal fraction were combined and dialyzed against the homogenization buffer (3–4 h at 5°C). The dialyzed mixture (approximately 3 mL) was then applied to a 1.5 × 2.5 cm DEAE (Sigma D8382) column which had been previously equilibrated with homogenization buffer. The citrate synthase activity was eluted form this column with a 0–1 M KCl gradient (Fig. 1B). One-millilitre fractions were collected and combined as described above. Following dialysis against homogenization buffer, the enzyme mixture was concentrated against solid sucrose and brought to 35% w/v glycerol in a final volume of approximately 3 mL. In this state, the citrate synthase activity was stable for at least 3 weeks when stored at 5°C. SDS gel electrophoresis of the solution showed the presence of one major and two minor bands with protein staining. Nevertheless, the intensity of the stained bands suggested that no more than 20–25% of the protein was not associated with citrate synthase.

Isoelectric focusing of heart citrate synthase

An LKB (ampholine) isoelectric focusing (IEF) column with a volume capacity of 110 mL was used in the determination of the isoelectric (IE) point of trout heart citrate synthase. The pH 3–10 gradient was obtained with the use of Sigma (A-5174) ampholine solution. Approximately 1 mL (5–6 units of activity) of crude heart supernatant was loaded halfway up the continuous density gradient (ampholine–sucrose). The initial voltage was set at 300 mV and 5 mA. Runs of 20, 14 and 6 h were made (see Results) at 5°C. One-millilitre fractions were taken at the end of the runs and the pH and citrate synthase activity were subsequently determined.

Assays and reagents

Citrate synthase activity was determined from the rate of appearance of reduced 5,5'-dithiobis-(2-dinitrobenzoic acid) (DTNB), which was monitored at 412 nm with a Gilford-240 recording spectrophotometer. Optimal assay conditions at 20°C and 20 mM Tris base were determined to be pH 8.5, 100 μM DTNB, 50 μM OAA, and 75 μM ACoA. The enzyme reaction was initiated by adding the purified enzyme preparation to 1 mL of the reaction cocktail. The addition of 2 μL (containing 110 ng protein) of the purified enzyme extract produced an optical density change of approximately 0.03/min. The molar absorption coefficient for DTNB is 13 600 (Srere 1972).

The concentration of protein in the extracts was measured by the method of Bradford (1976), using the BioRad Laboratories prepared reagent for Coomassie blue binding and bovine gamma globulin as the standard. All biochemicals and reagents were of analytical grade and were purchased from either Sigma Chemical Company, St. Louis, Missouri, or Boehringer–Mannheim, Montréal, Quebec. All solutions were made up in double-distilled deionized water.

Citrate synthase Arrhenius plot

Reaction rate determinations were made as described above, except that the reaction medium was buffered by 25 mM HEPES. HEPES media were made at 25°C at either pH 7.05 or pH 7.80 to reflect estimated in vivo values of myocardial 'cytosolic' and 'mitochondrial' pH, respectively. HEPES buffer was chosen so that the increase in pH of the reaction medium with decreasing temperature (dpH/dT°C HEPES = −0.014) would be consistent with that predicted by the alphastat hypothesis (dpH/dT°C = −0.016 to −0.018), as described by Reeves (1977). In one series of determinations the pH of the reaction medium
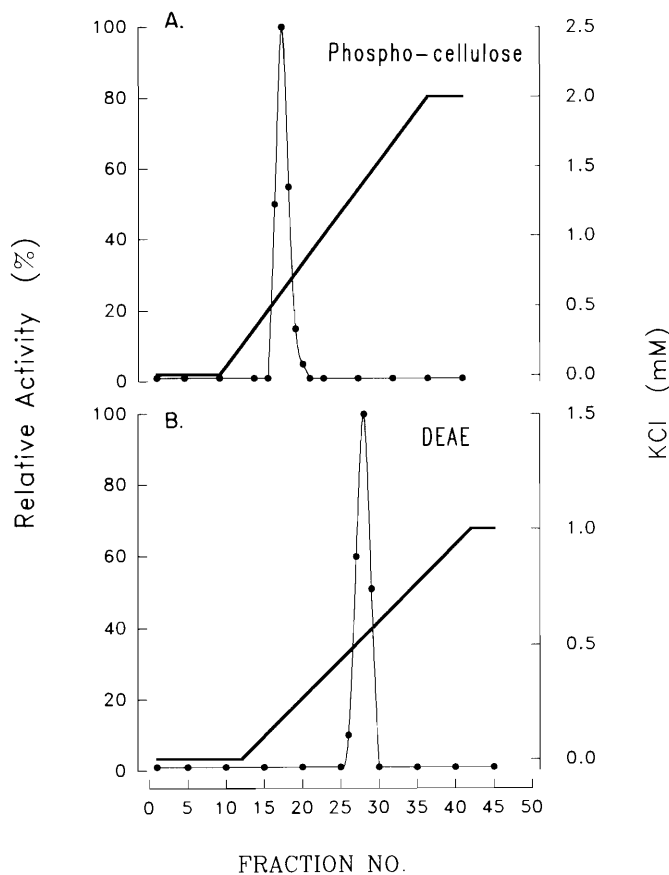

FIG. 1. Elution profiles of trout heart citrate synthase observed during column purification. Following an initial purification step using polyethylene glycol precipitation, the enzyme extract was applied to the cation-exchange column. The citrate synthase activity was eluted with a 0–2 M KCl gradient and collected in 1-mL fractions. Appropriate fractions were pooled and dialyzed against the phosphate buffer prior to application to the anion-exchange column. The enzyme activity was eluted from the latter column with a 0–1 M KCl gradient. These fractions were taken and pooled as described above. Both columns were loaded with approximately 3 mL protein solution and eluted at 1 mL · min^{-1} at 25°C.

was allowed to increase from either 7.05 ('cytosol') or 7.80 ('mitochondria') as the temperature was lowered from 25°C. In another series, HEPES solutions were made for use at each 5°C increment over the 0–25°C temperature span, so that the pH, either 7.05 ('cytostat') or 7.80 ('mitostat'), would be constant at each temperature. This was achieved by simply taking into account the temperature dependence of the pH of HEPES (see above) and compensating for this dependence when the solutions were prepared at 25°C.

The 'cytosolic' pH, 7.05, was arrived at by estimating the intracellular pH of teleost cardiac tissue in vivo at 25°C (calculated from the relationship given by Walsh and Moon 1982). An estimate of 'mitochondrial' pH was then made on the basis of data provided by Anderson et al. (1987), which describe the cytosolic pH (7.0) and the mitochondrial pH (7.8) of isolated rat hepatocytes incubated in vitro.

The cuvettes and their contents were brought to temperature by means of a circulating water bath heater/refrigerator coupled to a water jacket style cuvette holder. The maximal activity of the enzyme was measured at incrementally lower temperatures under saturating substrate conditions.

Statistical treatment

All values are given as the mean ±1 SEM unless indicated otherwise. Two-sample t-tests were used to determine the significance of differences ($p < 0.05$ served as the fiducial limit).

TABLE 1. Citrate synthase activity (μmol · min^{-1} · g^{-1} wet wt.) in rainbow trout tissues

Heart	30.8±1.8
White muscle	4.00±0.33
Liver	3.86±0.31
Gill	2.54±0.30
Red blood cells	0.12±0.01*

NOTE: Activities were determined at pH 8.5 and 20°C on the crude supernatants. Acetyl Co-A and oxaloacetic acid were present in excess at concentrations of 75 and 50 μM, respectively. The values above are the mean ± 1 SE of determinations on samples collected from four different fish. *From Ferguson and Storey 1991.

Results

Citrate synthase activity in crude preparations of various trout organs

The maximal activity of citrate synthase observed in the supernatant of crude homogenate solutions of trout heart was 30.8 ±1.8 ($n = 4$) units per gram wet weight of tissue at 20°C, pH 8.5. This value agrees well with previously reported levels of citrate synthase activity in the trout heart (MacIntyre and Driedzic 1981; Alp et al. 1976). Maximal activities of citrate synthase from various other organs were also determined and are listed in Table 1.

Purification and IEF of trout heart citrate synthase

Trout heart citrate synthase was purified 70-fold by a simple three-step treatment of the crude homogenate supernatant (Table 2). Following an initial PEG fractionation, citrate synthase was further purified by ion-exchange chromatography. The enzyme eluted with KCl from both phosphocellulose and DEAE columns as a single peak of activity (Fig. 1). In addition, citrate synthase activity was evident as a single peak following IEF (pI = 4.95, data not shown). These observations suggest that a single isozymic form of the enzyme was present.

Citrate synthase from rainbow trout heart was rather sensitive to IEF manipulation. Indeed, a 100% loss of activity was observed following 20-h runs at 5°C. Incubations over a much shorter time period (i.e., 6 h) gave IEF values in the range 5.4–5.5 and reduced the loss of enzyme activity to 60%. However, peak profiles and pH gradients were unsatisfactory. Fourteen-hour incubations resulted in an 80% loss of enzyme activity yet produced a sharp peak and allowed a good pH gradient to be established. The IE point indicated by the latter runs was 4.95 and was considered the best estimate for trout heart citrate synthase under the conditions of this study. Citrate synthase from the rainbow trout liver is likewise sensitive to similar IEF procedures; the estimated IE range for liver citrate synthase is 5.1–5.8, using similar IEF conditions and durations (Hochachka and Lewis 1970). This apparent instability of trout citrate synthase should be considered in future studies in which IEF characterization is planned.

Kinetic characteristics of purified citrate synthase

The maximal activity of partially purified trout heart citrate synthase was obtained in vitro with 50 μM OAA and 75 μM ACoA at 20°C in Tris–HCl buffer, pH 8.5. The relative substrate affinities of the enzyme are reflected by the micromolar K_m values for OAA and ACoA, which are listed in Table 3. Hill analyses of this enzyme showed that it followed hyperbolic Michaelis–Menten substrate saturation kinetics (Fig. 2, Table 3).

Effects of varying pH and temperature on maximal activity

To characterize the dependence of trout heart citrate synthase activity on ambient pH, we evaluated the activity of the enzyme under conditions of varying pH (6–9.5) and saturating substrate conditions at 20°C. The pH activity profile which we obtained shows that the optimum for this enzyme lies at pH 8.5 (Fig. 3). Acidification of the reaction medium resulted in significant loss of enzyme activity. Indeed, at pH 6, citrate synthase activity was reduced to 20% of the value observed at pH 8.5.

The pH of the reaction medium was observed also to influence the effect of temperature on citrate synthase activity. The reaction temperature was varied over the physiological range for this species (0–25°C) and the activity of citrate synthase was determined under conditions of saturating substrate concentration (Fig. 4). It is evident from the slopes of the lines in the Arrhenius plot that as the temperature was reduced, the maximal activity of citrate synthase was also reduced. It is equally evident that with decreasing temperature, the maximal activity of trout heart citrate synthase deviated most from the value observed at 25°C when the enzyme was incubated in the 'cytosolic' (i.e., low-pH) buffers. Another feature of the Arrhenius plot is the discrepancy between the slopes of the two 'mitochondrial' preparations. The shallower of these slopes corresponds to the preparations in which pH was allowed to increase as temperature deceased.

Activation energies were calculated from the slopes of the data presented in the Arrhenius plot (Fig. 4). The 'cytosol' and 'cytostat' relationships provided activation energy values of 15.9 and 15.7 kcal/mol, respectively (1 kcal = 4.1855 kJ). The activation energy of trout heart citrate synthase calculated from the 'mitostat' data, on the other hand, was 10.6 kcal/mol. The lowest estimate of trout heart citrate synthase activation energy, 7.8 kcal/mol, arose from the data described by the shallow slope of the 'mito' relationship. This is comparable to the value of 8.8 kcal/mol determined for trout liver citrate synthase from 5 to 30°C, pH 7.5 (Hochachka and Lewis 1970). It is clear that the pH of the reaction medium has a significant effect on the observed relationship between citrate synthase activity and temperature, as well as on the activation energy calculated from such relationships.

Modification of citrate synthase activity by various metabolites

In addition to the effects of temperature and pH, the influence of several metabolites on trout heart citrate synthase activity was evaluated. Though citrate synthase is not susceptible to substrate inhibition (Fig. 2), several biochemical compounds, including citrate, were found to inhibit enzyme activity at 20°C, pH 8.5. The I_{50} values for these metabolites are presented in Table 4.

The nature of the inhibition of citrate synthase by the various metabolites listed in Table 4 was further evaluated using Lineweaver–Burk reciprocal plots. Citrate synthase activity was observed under conditions of varying OAA concentration in the presence of citrate, α-ketoglutarate, and ATP (Fig. 5). Activity of this enzyme was inhibited by 50% in the presence of ~3 mM citrate (Table 4). This contrasts with the liver isozyme, which is not inhibited by citrate at levels up to 1 mM (Hochachka and Lewis 1970). The form of the citrate inhibition ($K_i = 8.80$ mM) was strictly competitive in relation to OAA. As a result, the calculated maximal velocity of the enzyme was not diminished even in the presence of high levels of the product (Fig. 5A, Table 5). As in the case of rat liver citrate synthase ($K_i = 10$ mM, Srere et al. 1973), α-ketoglutarate ($K_i = 13.95$ mM) inhibited trout heart citrate synthase in a competitive manner

TABLE 2. Summary of the partial purification of trout heart citrate synthase

Step	Total activity (units)*	Yield (%)	Specific activity (units/mg)	Degree of purification
1. 30 000 × g supernatant	14.4	100	0.258	1.0×
2. Polyethylene glycol	9.0	63	0.649	2.51×
3. Phosphocellulose	4.0	28	3.914	15.2×
4. DEAE	3.0	21	18.21	70.6×

*One unit of enzyme activity is that which converts 1 μmol oxaloacetic acid to 1 μmol citrate in 1 min at 20°C.
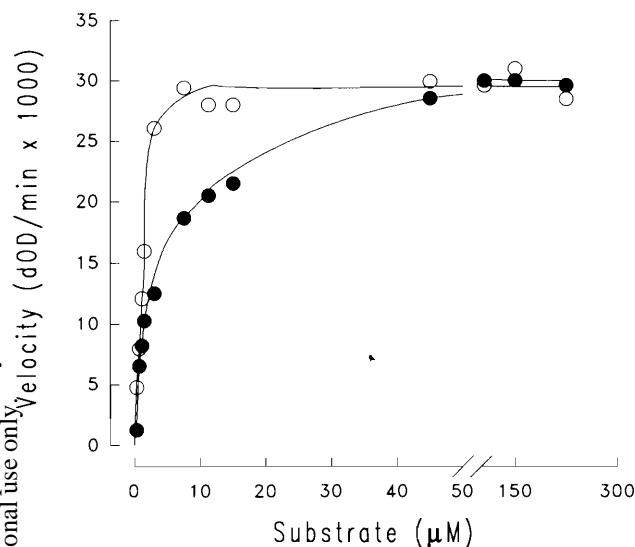
FIG. 2. Saturation curves for partially purified trout heart citrate synthase. Velocity is presented in terms of the change in optical density (OD) observed per minute (dOD/min). The velocity versus substrate concentration curves are hyperbolic, regardless of whether ACoA (●) or OAA (○) is varied. Assay conditions were as follows: 100 μM DTNB and either 50 μM OAA and various ACoA concentrations or 60 μM ACoA and various OAA concentrations in 1 mL 20 mM Tris buffer, pH 8.5, at 20°C. Approximately 2 μL of the partially purified enzyme preparation (~110 ng protein) was added to initiate the reaction.

TABLE 3. Apparent K_m value (μM) and Hill coefficient (n_H) for substrates of heart citrate synthase incubated at pH 8.5 and 20°C

OAA	
K_m	1.65±0.21
n_H	1.25±0.04
ACoA	
K_m	3.91±0.66
n_H	0.99±0.07

NOTE: All values are given as the mean ± 1 SE of three independent determinations. For determinations of the apparent K_m of OAA, ACoA was held constant at 60 μM. OAA was held at 50 μM during the determination of the apparent K_m of ACoA. For other assay conditions see Fig. 2.
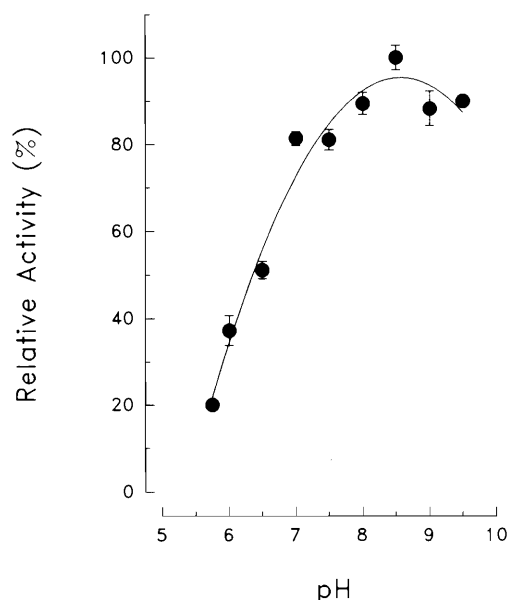

FIG. 3. Effect of pH on the activity of citrate synthase. Both ACoA and OAA were maintained at saturating levels over the range of pH used. Other assay conditions were as described in Fig. 2, except that the assay media were buffered with 20 mM KPO₄.

(Fig. 5B, Table 5). In contrast, ATP interacted noncompetitively with OAA to inhibit the enzyme's activity (K_i = 1.04 mM). At saturating levels of ACoA, ATP inhibited heart citrate synthase activity through depression of the maximal velocity and not through any alteration of the enzyme's affinity for OAA (Fig. 5C, Table 5).

While noncompetitive inhibition by ATP was seen with respect to OAA, competitive inhibition of trout heart citrate synthase by ATP was observed with respect to ACoA (K_i = 0.382 mM; Fig. 6A, Table 6). Similar bimodal inhibition by ATP is reported for trout liver citrate synthase (Hochachka and Lewis 1970). Like ATP, NADH (K_i = 0.451 mM; Table 6, Fig. 6B) inhibits citrate synthase in a competitive manner with respect to ACoA as well. The fact that ATP and NADH act as competitive inhibitors has been cited as partial proof that a general nucleotide binding site exists on this enzyme (Srere 1972). Despite this, we could find no inhibition of trout heart citrate synthase by AMP when this nucleotide was added to concentrations up to 5 mM (data not shown). This finding is in agreement with studies of the pig heart enzyme (Kosicki and Lee 1966). Furthermore, NAD (Table 4) did not inhibit citrate synthase activity.
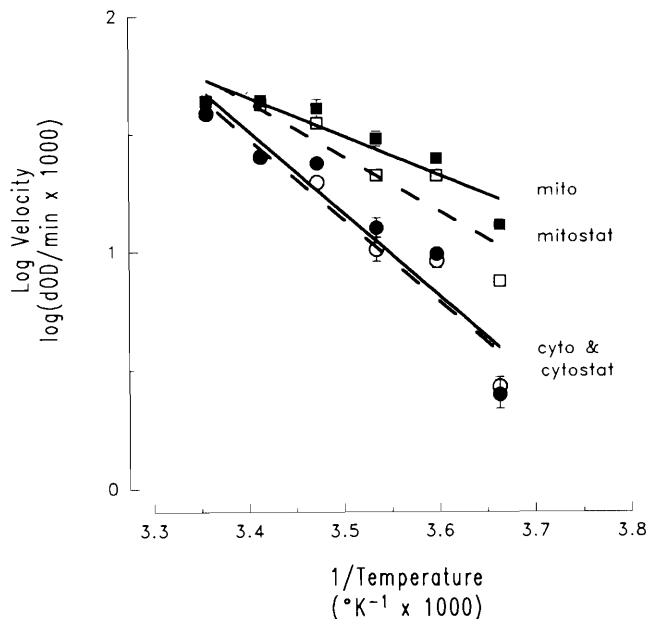
FIG. 4. Arrhenius plot of partially purified trout heart citrate synthase. Maximal enzyme activities were determined over a 0–25°C temperature span in 25 mM HEPES. The buffers were made up to pH 7.05 ('cytosol') or pH 7.8 ('mitochondria') at 25°C. 'Mitostat' ($\square$– – –$\square$) and 'cytostat' ($\bigcirc$– – –$\bigcirc$) preparations were maintained at pH 7.05 or 7.8, respectively, throughout the temperature range. The pH of the 'mito' ($\blacksquare$——$\blacksquare$) and 'cyto' ($\bullet$——$\bullet$) preparations, on the other hand, was allowed to increase with decreasing temperature, as demanded by the temperature dependence of HEPES ($dpH/dT°C = -0.014$). Linear regression analysis indicated that the four relationships were adequately represented by linear curve fitting ($r \geq 0.95$). There was no significant difference between the cyto and cytostat slopes (-3.49 ± 0.46 and -3.45 ± 0.37, respectively), whereas both were significantly lower than the slope of either the mito (-1.72 ± 0.39) or mitostat (-2.32 ± 0.48) relationships. Each point is the mean $\pm$ 1 SE of three determinations.

TABLE 4. I_{50} values for some metabolic effectors of trout heart citrate synthase

Metabolite	I_{50} (mM)
NADH	3.90 ± 0.54
NAD$^+$	No effect*
Citrate	3.17 ± 0.28
α-ketoglutarate	7.09 ± 0.26
ATP	0.272 ± 0.056
AMP	No effect*
ATP:Mg	2.27 ± 0.28

NOTE: I_{50} values were determined with ACoA and OAA present at 5.0 μM each (pH 8.5, 20°C). Other assay conditions are described in Fig. 2. The values listed above are the mean $\pm$ 1 SE of three determinations.

*No change was seen in enzyme activity with either NAD$^+$ or AMP present at concentrations ≤5 mM.

Discussion

It is evident that the trout heart possesses abundant citrate synthase activity (Table 1), a property that reflects the importance of aerobic metabolism to teleost cardiac function *in vivo* (e.g., Nielson and Gesser 1984; Lanctin *et al.* 1980). Citrate synthase isolated from the trout heart shows a high affinity for its substrates, as evidenced by the low micromolar range K_m values
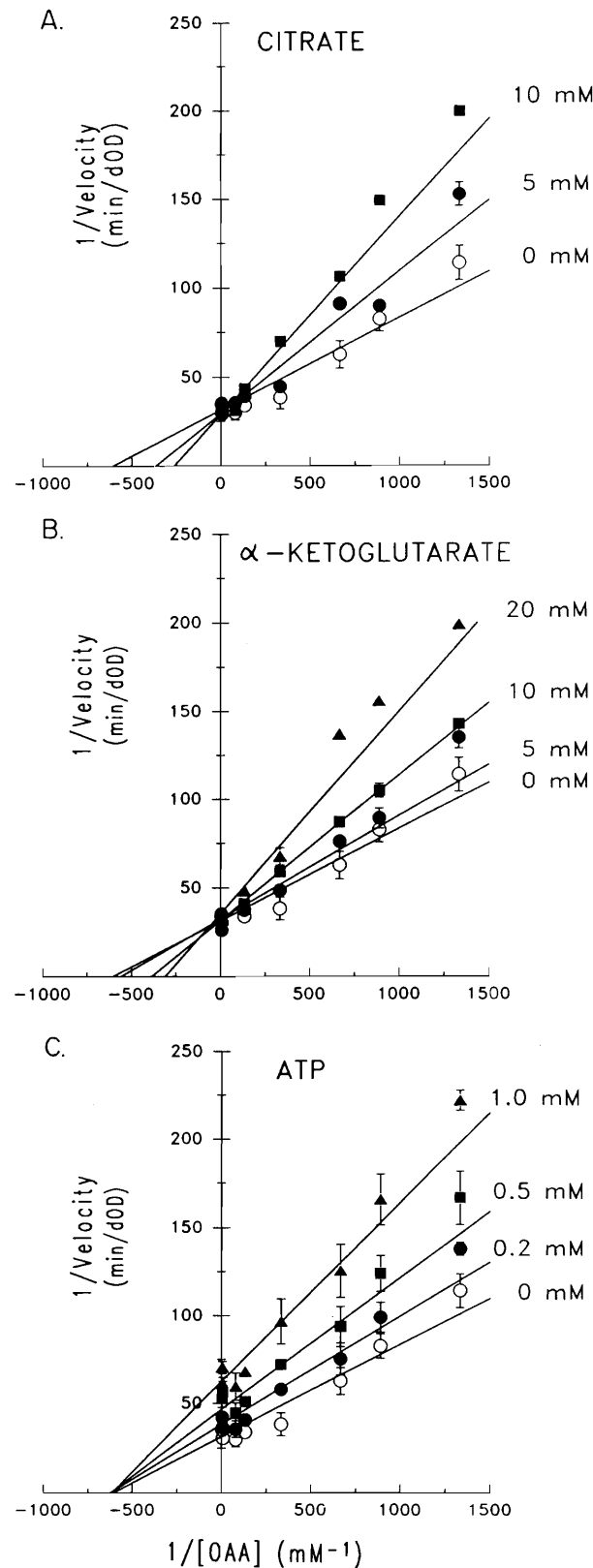
FIG. 5. Lineweaver–Burk reciprocal plots of citrate synthase activity in respect to OAA in the presence of various inhibitors. The reaction conditions are described in Fig. 2, except that the appropriate inhibitor was present in the reaction media at the designated concentration prior to addition of citrate synthase. (A) Competitive inhibition by citrate. (B) Competitive inhibition by α-ketoglutarate. (C) Noncompetitive inhibition by ATP. Each point is the mean $\pm$ 1 SE of at least three determinations (see also Table 5).

TABLE 5. Effect of inhibitors on the K_m value for OAA and other kinetic properties of trout heart citrate synthase

Effector	K_m (μM)	V_{max} (dOD/min)	n_H
Control	1.65	0.032	1.25
5 mM citrate	2.74*	0.036	1.08*
10 mM α-ketoglutarate	2.59*	0.032	1.01*
0.5 mM ATP	1.60	0.021*	1.22

NOTE: The velocity of the enzyme reaction is reported in terms of the change in optical density observed per minute (dOD/min). Assay media contained 60 μM ACoA and varying concentrations of OAA (see Fig. 2 for other assay conditions). Each value is the mean of three determinations.

*Significantly different from control value, two-sample t-test, $p <$ 0.05. The specific concentrations of inhibitors presented above were chosen arbitrarily to approximate I_{50} values (see Fig. 5).

TABLE 6. Effect of inhibitors on the K_m value for ACoA and other kinetic properties of trout heart citrate synthase

Effector	K_m (μM)	V_{max} (dOD/min)	n_H
Control	3.90	0.031	0.99
0.5 mM ATP	17.3*	0.035	0.83*
4 mM NADH	16.2*	0.036*	1.00

NOTE: The assay media contained 50 μM OAA and varying concentrations of ACoA (see Fig. 2 for other assay conditions). Each value is the mean of three determinations.

*Significantly different from control value, two-sample t-test, $p <$ 0.05. The specific concentrations of inhibitors presented above were chosen arbitrarily to approximate I_{50} values (see also Fig. 6).

(Table 3). These values correspond very well to K_m values determined for mammalian heart citrate synthases (Srere 1972). However, apparent K_m values reported for the trout liver enzyme are much higher than those for the trout heart enzyme: approximately 18 and 75 μM for OAA and ACoA, respectively, at 20°C, pH 7.5 (Hochachka and Lewis 1970). It would appear that the heart enzyme has a much higher substrate affinity than the liver citrate synthase under the conditions of these studies. To what degree the difference in experimental pH contributes to the large discrepancies in trout liver and heart K_m values is not known. It is possible, however, that these values reflect differences in the ranges of substrate concentration in the respective cells (Srere 1972).

Trout heart citrate synthase activity is dependent to a very large degree on the pH of the reaction medium (Fig. 3). The mitochondrial pH is generally very much higher than the cytosolic pH of vertebrate cells. For example, Andersson et al. (1987) reported a pH gradient of nearly 1 full unit across the mitochondrial membrane of rat hepatocytes. Eel cardiac intracellular pH at 20°C is in the vicinity of pH 7.15 (Walsh and Moon 1984). At this pH, trout heart citrate synthase activity is approximately 65–70% of the maximal value observed at pH 8.5 in vitro (Fig. 3). If it is presumed that citrate synthase of the trout heart is restricted to the relatively alkaline intramitochrondrial compartment, as is the case in trout hepatocytes (Walton 1985), peak activity of this enzyme at a pH in the alkaline range (i.e, pH 8.5) would indeed be appropriate for ensuring optimum activity in vivo.

In addition to pH, we were interested in examining the influence of temperature on citrate synthase activity, given the importance of aerobic metabolism in cold adaptation by ectotherms. The influence of pH on the response of citrate synthase activity to varying temperatures over the physiological range for rainbow trout (0–25°C) was determined under conditions of saturating substrate concentrations (Fig. 4). It is evident from the slopes of the lines in Fig. 4 that as the temperature falls, maximal activity of citrate synthase is maintained closer to that observed at 25°C when it is incubated in the 'mitochondrial' buffers. Thus, as the temperature changes, swings in citrate synthase activity are dampened at the higher pH values. Another feature of the Arrhenius plot is the discrepancy between the slopes of the 'mitochondrial' preparations. The shallower of these slopes corresponds to the preparations in which pH increased as temperature decreased. Indeed, the intracellular dpH/dT relationship exhibited by the teleost heart does conform to the alphastat model of acid–base regulation (Walsh and Moon 1982). It is
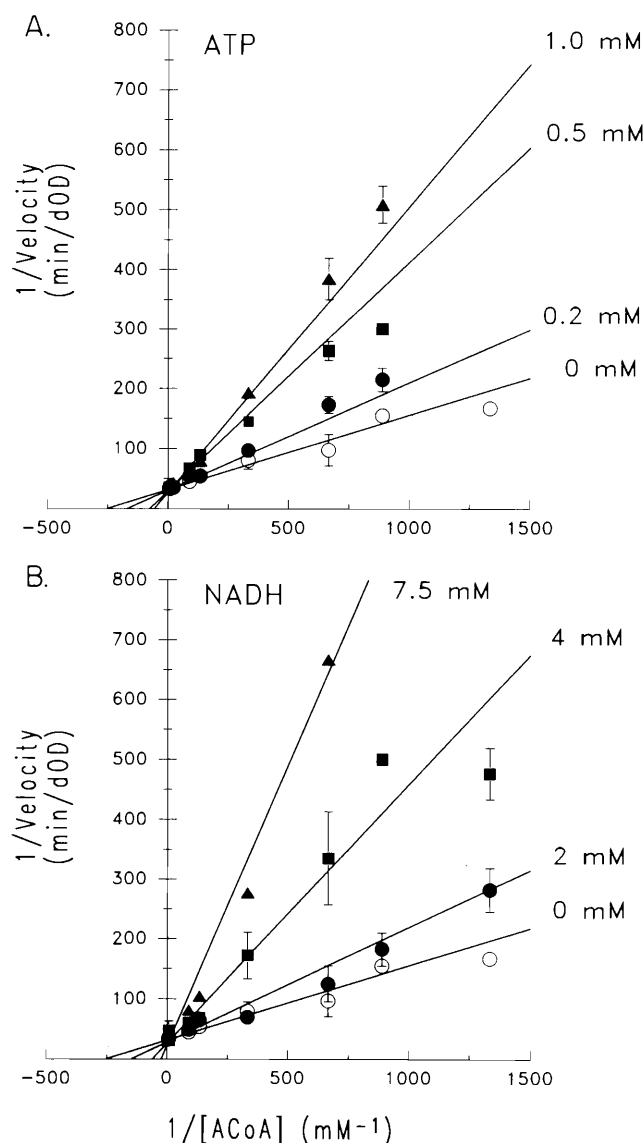

FIG. 6. Lineweaver–Burk reciprocal plots of citrate synthase activity with respect to ACoA in the presence of various inhibitors. The reaction conditions are described in Fig. 2, except that the appropriate inhibitor was present in the reaction media at the designated concentration prior to addition of citrate synthase. (A) Competitive inhibition by ATP. (B) Competitive inhibition by NADH. Each point is the mean ± 1 SEM of at least three determinations (see also Table 6).

likely, therefore, that the aforementioned *in vitro* condition (i.e., high pH varying with temperature: $dpH/dT°C$ HEPES $= -0.014$) most closely approximates the conditions in which trout heart citrate synthase must function *in vivo*.

Provided the 'mitochondrial' preparations represent a good approximation of the conditions in which citrate synthase functions *in vivo*, and that citrate synthase activity is indicative of cardiac oxygen consumption, one would expect the activity profile of both to be similarly affected by changing temperature. Indeed, between 5 and 15°C, the 'mitochondrial' preparations of citrate synthase exhibit Q_{10} relationships of 1.65 ± 0.15 ($n = 3$) and 1.67 ± 0.04 ($n = 3$) for 'mito' and 'mitostat' preparations, respectively. These values are remarkably similar to that exhibited over the same temperature range by the myocardial oxygen consumption associated with maximum sustained swimming speed in rainbow trout ($Q_{10} = 1.7$, calculated from data available in Graham and Farrell 1990). On the other hand, the more acidic 'cytosolic' preparations of citrate synthase display significantly higher Q_{10} values than their 'mitochondrial' counterparts (Q_{10} values at 5–15°C for 'cyto' and 'cytostat' preparations are 2.42 ± 0.06 ($n = 3$) and 2.17 ± 0.14 ($n = 3$), respectively).

The results obtained from the Arrhenius plot of trout heart citrate synthase are interesting, as they suggest that cellular pH set points, as well as cellular dpH/dT properties, are important factors which influence the response of citrate synthase (and presumably the TCA cycle) to temperature perturbations. Hochachka and Lewis (1970) have found that at subsaturating substrate levels (in which case K_m will play a larger role in determining the enzyme activity than at saturating substrate levels), the K_m values for OAA and ACoA of trout liver citrate synthase isozymes decrease with decreasing temperature. Thus, there appears to exist yet another strategy by which trout citrate synthase activity is stabilized in the face of thermal changes.

These data represent an exciting avenue of investigation into the possible relationships between the control of citrate synthase activity and the relatively low Q_{10} values reported for ectotherm aerobic metabolism (Hochachka and Lewis 1970; Hochachka and Somero 1984). Indeed, a recent comparison of antarctic and temperate zone marine fishes found that this enzymic property (i.e, low thermal sensitivity) may underlie the selective expansion of aerobic metabolism to support activity by ectotherms inhabiting polar waters (Crockett and Sidell 1990). Factors related to the thermal sensitivity of citrate synthase, such as the cell and (or) organelle pH regulatory properties, may indeed be instrumental in determining the ability of an organism to exploit low-temperature environments.

Cardiac citrate synthase activity is not solely dependent on temperature and pH but is also modified by various cellular biochemical compounds. These metabolites are important indicators of cellular activity/energy status, given that the levels of these metabolites will change as the metabolic demands of cardiac function change. Given the effects of NADH and NAD$^+$ (Table 4) on citrate synthase activity *in vitro*, it would appear that the redox state of the heart could influence the activity of cardiac citrate synthase, and the TCA cycle, *in vivo*. Recent studies on the mammalian heart place estimates of intracellular NADH concentrations at approximately 50 μM or less (Bessho *et al.* 1989). It is clear that the sensitivity of the trout enzyme to NADH inhibition *in vitro* does not extend to this low level insofar as regulatory potential is concerned (Fig. 6, Tables 4 and 6). However, as pointed out by Srere (1972, 1975), factors such

as the compartmentalization of citrate synthase on the mitochondrial membrane as well as the microenvironments of metabolite concentrations that exist within the mitochondria may allow for such regulation *in situ*. Similar circumstances may be related to the relatively high concentrations of citrate and α-ketoglutarate required to regulate the trout heart enzyme *in vitro* (Table 4) compared with the intracellular levels commonly found in animals *in vivo* (Williamson and Brosnan 1974).

Inhibition of trout heart citrate synthase by citrate (Fig. 5A) offers a typical example of enzyme regulation through product inhibition. ATP may be considered an end-product as well, since the ultimate consequence of TCA cycle activity is ATP production. ATP appears to be a much more potent inhibitor of trout heart citrate synthase than of trout liver citrate synthase. Hochachka and Lewis (1970) observed similar inhibition of trout liver citrate synthase by ATP but at nucleotide levels typically 5 times those used in this study. The K_i value of ATP for the trout heart enzyme (0.38 mM with respect to ACoA) corresponds quite well to values reported in the literature for the mammalian enzyme (e.g., Srere *et al.* 1973). Evident from the data in Table 4 is the ameliorating effect exerted by an equimolar amount of Mg^{++} on ATP inhibition. This effect is also seen, for example, with pig heart citrate synthase (Kosicki and Lee 1966). This is presumed to be because the MgATP complex is noninhibitory (Srere 1972). Despite this, ATP:Mg remains inhibitory to trout heart citrate synthase, albeit at concentrations 10 times those required to get a similar effect with ATP alone.

The data presented here with respect to ATP:Mg and AMP *in vitro* would appear to support a role for ATP and (or) adenylate charge in regulating citrate synthase in the trout heart *in vivo*. Typical intracellular ATP levels found in perfused fish hearts (approximately 1.5 mM; Turner and Driedzic 1980) and rainbow trout ventricular strips (approximately 3–5 mM; Hansen and Gesser 1987) are in general agreement with the ATP:Mg levels required to produce (50%) inhibition of rainbow trout heart citrate synthase *in vitro* (Table 4). Thus, in this case the levels of the metabolite in the cell correspond well to the range required to regulate the enzyme's activity *in vitro*.

Further work will better define the existing correlates between the aerobic metabolism/function of the ectotherm heart and cellular levels of metabolites such as those described above. Another important group of metabolic modulators of vertebrate citrate synthases are the fatty acid derivatives (e.g., Fritz and Halperin 1973). How the regulation of these metabolites and (or) their associated pathways influences the interplay of enzyme thermal sensitivity and cell pH *in situ* remains to be established. Nevertheless, the potential for regulation of trout heart aerobic metabolism during physiological stresses such as thermal perturbation does appear to exist via adjustments in cellular pH and the levels of various metabolites, as demonstrated here *in vitro*.

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