SUMMARY

Citrate synthase occurs in two kinetically distinguishable forms, termed C and W in cold and warm acclimated trout, respectively. Oxalacetate and acetyl-CoA saturation curves for both enzymes are hyperbolic and do not show any substrate inhibition. The apparent $K_m$ values for the C form increase with temperature above 10°C. At saturating (0.1 mM) levels of oxalacetate, the $K_m$ of acetyl-CoA is 0.05 mM at 10°C, about one-fifth the value at 35°C. At saturating levels of acetyl-CoA, the $K_m$ of oxalacetate is 7 μM at 10°C, about one-fourth the value at 35°C. These values at low temperature are similar to the $K_m$ constants for the W form below 15°C, but above 15°C, the $K_m$ values for the W form are distinctly lower than for the C form. In the upper biological temperature range for both cold and warm acclimated trout, the Q10 for citrate synthase is about 1.0 at low substrate concentrations because the increase in $K_m$ compensates for the increasing thermal energy. At saturating substrate concentrations, the temperature characteristics of the two enzymes are the same, and the calculated activation energies are 8.8 kcal per mole.

Both C and W forms of citrate synthase are strongly inhibited by ATP, which increases the $K_m$ of acetyl-CoA (5-fold at pH 7.5 and 22°C) but does not alter the calculated $V_{max}$. ATP inhibition is noncompetitive with respect to oxalacetate, and the oxalacetate saturation curve remains hyperbolic in the presence of ATP. The $K_i$ of ATP for the C form increases dramatically with temperature above 15°C, while the $K_i$ of the W form is thermally insensitive to nearly the lethal limit for trout.

The calculated $V_{max}$ values for both C and W forms increase 4-fold between pH 6.5 and 8.5, but the $K_m$ values are rather insensitive to pH changes. The $K_i$ of ATP, on the other hand, increases 4-fold between pH 6.5 and 8.5. Since the pH in poikilothermic tissues is relatively well documented at several levels of organization (7), our experimental organism, Salmo gairdneri, was obtained from a local hatchery. The animals used were all from a small brood stock; hence, genetic variability was substantially lower than in wild stock rainbow trout. The trout were acclimated to 2 and 18°C by holding them at these temperatures for at least 4 weeks. Photoperiod and feeding rates were similar for both acclimation groups.

Methods and Materials

Experimental Animals—The salmonids are generally eurythermal, and their thermal relations as a group are relatively well documented at several levels of organization (7). Our experimental organism, Salmo gairdneri, was obtained from a local hatchery. The animals used were all from a small brood stock; hence, genetic variability was substantially lower than in wild stock rainbow trout. The trout were acclimated to 2 and 18°C by holding them at these temperatures for at least 4 weeks. Photoperiod and feeding rates were similar for both acclimation groups.

Enzyme Assay—Citrate synthase activity was followed by measuring the increase in absorbance at 412 nm resulting from the reaction of CoA—SH, liberated in the enzymatic reaction, with DTNB (8). The assay cuvette (1-em light path) contained 25 μM DTNB, varying concentrations of acetyl-CoA and oxalacetate, and the acclimation temperature of the organism. Over acclimation periods of 1 to several weeks, the respiration of many poikilothermic systems is adjusted, often in a manner that compensates for the new thermal regime (see Reference 1 for a recent review). Short term or immediate compensation of aerobic metabolism in poikilothermic tissues, less commonly observed, is also known. Thus, anaerobic, pyruvate, and acetate oxidation rates by fish tissues and by mitochondria prepared from intertidal organisms are stable over most of each species' thermal range (2-4). Similarly, O2 consumption by tuna muscle minces is insensitive to temperature between 5 and 35°C (5). Although the functional advantages of such short term and long term thermal compensations are clear, underlying enzymic mechanisms are not.

In this context, we initiated a study of the properties of citrate synthase (citrate oxalacetate-lyase (CoA-acetylating), EC 4.1.3.7) from liver tissue of cold and warm acclimated trout. In other organisms examined, the enzyme occurs as a single molecular species strongly modulated by the adenylates (6). In the trout, we found that liver citrate synthase occurs in two kinetically distinguishable forms. One form occurs in cold (2°C) acclimated organisms, while an alternate form of citrate synthase occurs in the liver of warm (18°C) acclimated organisms. In the upper biological temperature range for both cold and warm acclimated fish, increases in the apparent Michaelis constants compensate for increasing temperatures; in consequence, at low substrate concentrations, the $Q_{10}$ of the reaction catalyzed by each variant is reduced.

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FIG. 1. Isoelectric resolution of liver citrate synthases from cold and warm acclimated trout. Isoelectrofocusing was performed for 12 hours at 4°C with synthetic ampholytes, forming a pH 3 to 10 gradient. Initial conditions: 300 volts, 5 ma. The enzymatic activity (V) of each fraction is plotted on the ordinate as a percentage of the peak activity.

acetate, and enzyme in a final volume (2.0 ml) of 0.1 M Tris-HCl buffer, usually at pH 7.5. All assay reagents were made up in Tris buffer at the desired pH. After mixing the reagents in a cuvette of 1-cm light path and allowing time for thermal equilibration, the reaction was started by addition of one of the substrates. In most cases, enough enzyme was added to produce an increase of 0.2 to 0.4 absorbance unit per min. Change in optical density was measured in a Unicam SP 800 recording spectrophotometer (Norelco Division, Philips Electronic Instruments, Mount Vernon, New York). Cuvette temperatures were controlled to within 0.1°C between 2 and 40°C by a Lauda constant temperature bath and circulator (Brinkman Instruments, Inc., Westbury, New York). When necessary, care was taken to set the pH of Tris-HCl buffer at the assay temperatures, since the pK of Tris is temperature-dependent.

Preparation of Enzyme—Freshly excised or frozen tissues (maintained at about -10°C) were taken up in 0.1 M Tris-HCl buffer at pH 7.5, and 20% homogenates were prepared with a VirTis homogenizer. The homogenate was centrifuged at about 5000 × g for 90 min, and the pellet, which contains only about 10% of the total citrate synthase activity, was discarded. The supernatant was brought to 33% saturation with ammonium sulfate (200 g per liter), stirred for at least 1 hour, and centrifuged. The precipitate, which showed no activity, was discarded. The supernatant was brought to 70% saturation with ammonium sulfate (472 g per liter) and again stirred for 1 hour. After centrifugation, the precipitate, which showed all of the remaining citrate synthase activity, was taken up in 0.1 M Tris-HCl buffer, pH 7.5. After dialysis, the preparation was used directly as a source of citrate synthase activity. The enzyme was stable upon freezing for at least 3 to 4 weeks.

Isoelectrofocusing—The electrofocusing column had a volume capacity of 110 ml and was equipped with double cooling jackets (LKB-Produkter AB, Stockholm, Sweden). The column was held at 3°C by a refrigerated, constant temperature bath and circulator. The pH 3 to 10 gradient was achieved with ampholyte LKB 8141 (a mixture of polyaminopolycarboxylic acids). The cathode solution was routinely placed at the bottom of the column. The high speed supernatant solution of freshly prepared tissue homogenates, which was used as a source of enzyme, was layered on the sucrose-ampholyte about halfway up the column. Initial voltage was usually set at 300 volts; initial current was about 5 ma. Upon completion of the run, the column was drained at about 10 to 15 drops per min and collected with an LKB fraction collector. Fractions were assayed for citrate synthase activity as described above. The pH of each fraction was measured at 20°C with a Radiometer pH meter (Copenhagen, Denmark).

Reagents—All organic reagents (acetyl-CoA, oxalacetate, DTNB, Tris) were obtained from Sigma. All other compounds used were reagent grade.
RESULTS

Electrophoretic Forms of Citrate Synthase—In mammalian systems, citrate synthase occurs in at least two electrophoretically distinguishable forms, which are thought to represent oxidized and reduced states of a single protein species (9, 10). In the case of 2° acclimated trout, liver citrate synthase usually migrates as a single recoverable activity peak during isoelectrofocusing in pH 3 to 10 gradients (Fig. 1). For convenience, we refer to this form of the enzyme as the C form or the 2° enzyme. In 18° acclimated fish, citrate synthase also migrates as a single major peak, which we designate the W form of the enzyme. In short (10 hours) parallel runs, in which activity loss during electrofocusing is reduced, the C and W forms of the enzyme can be readily distinguished (Fig. 1). Longer (24 to 36 hours) electrofocusing indicates that the isoelectric points for both enzymes are below pH 5.0 but leads to about 90% loss of citrate synthase activity. We have no information on the nature of enzyme inactivation. Recoverable activity of both C and W forms is usually in single peaks, although minor shoulders are occasionally observed. The major and minor peaks may represent oxidized-reduced states of the enzyme or alternate isozymes, as suggested by the minor shoulder appearing in the 18° preparation in Fig. 1. Further studies should clarify the nature of these variants.

Temperature Effects on $V_{max}$ and on $K_m$ Values of Substrates.—In agreement with kinetic studies on other citrate synthases, substrate saturation curves for trout liver citrate synthases are hyperbolic at all temperatures examined (Figs. 2 and 3), and excess of substrates is not inhibitory. The apparent Michaelis constants of both substrates decrease at lower temperatures, however, so that at low substrate values (0.01 mM acetyl-CoA; 0.01 mM oxalacetate), the saturation curves, particularly for the C form, converge sharply at all temperatures. This effect
is seen more clearly when the $K_m$ values, determined by Lineweaver-Burk plots, are plotted against temperature (Fig. 4). It is evident that the apparent $K_m$ values of both C and W variants increase with temperature over the upper thermal range. The apparent $K_m$ values for acetyl-CoA and oxalacetate of the C form appear to be more temperature-dependent than those of the W form; the minimum $K_m$ values for the C form are about one-fifth the $K_m$ values at 35°. In the case of the W form, the minimum $K_m$ values of acetyl-CoA and oxalacetate are only about one-half the values at 35°. The minimum $K_m$ values occur at about 10°.

A consequence of the $K_m$-temperature relationship is the reduction of $Q_10$. At low concentrations of substrates, the decrease in apparent $K_m$ can compensate completely for reduced thermal energy. This is evident in Arrhenius plots for both the C and W forms of the enzyme under conditions of saturating (0.1 mM) oxalacetate concentrations and various acetyl-CoA concentrations (Fig. 5). Under these conditions above 10°, the $Q_{10}$ for the C form is about 1 at 0.025 mM acetyl-CoA and can be less than 1 at 0.01 mM acetyl-CoA. At saturating acetyl-CoA concentrations, the $Q_{10}$ is about 1.7 (Fig. 5). The activation energies for both enzymes (8.8 kcal per mole), calculated over the temperature range of 5-30°, are lower than the values for the mammalian enzyme. Arrhenius plots for the pig enzyme show a break at 20°; at temperatures below 20°, the activation energy is 13.7 kcal per mole, while above 20° it is about 9.7 kcal per mole (11). This complex behavior is not shown by the trout enzymes over the temperature range that we studied (Fig. 5), and the Arrhenius plots appear to be linear to temperatures beyond the lethal limit (25-27°) for salmonids (12).

Adenylate Modulation—As pointed out above, the reaction catalyzed by trout liver citrate synthases follows normal Michaelis kinetics with respect to both substrates. ATP is a potent inhibitor of the reaction, and the inhibition is competitive with respect to acetyl-CoA (Fig. 6). The $K_m$ of acetyl-CoA increases by about 4- to 5-fold at 5 mM ATP and 22°; under similar conditions, there is little if any effect upon the calculated $V_{max}$. These characteristics are similar to those of other citrate synthases (10, 13). Preliminary experiments indicate that, at higher concentrations (above 5 mM at 22°), ATP alters both the $K_m$ of acetyl-CoA and the $V_{max}$. In the presence of ATP, acetyl-CoA saturation curves remain hyperbolic, and the activation energy is not altered.

With respect to oxalacetate, ATP inhibition is noncompetitive (Fig. 7), again in agreement with studies of the enzyme from other organisms (6). Also, oxalacetate saturation curves remain hyperbolic in the presence of ATP (Fig. 7).

AMP and ADP also inhibit trout liver citrate synthase. AMP is notably less effective; in a typical experiment, 8 mM AMP reduced the activity to only 80% of control. ADP, on the other hand, is nearly as effective an inhibitor as ATP; similar concentrations of ADP or ATP yield comparable percentages of inhibition. ADP inhibition is also competitive with respect to acetyl-CoA and noncompetitive with respect to oxalacetate. We have not examined the ADP inhibition in further detail and have assumed that its behavior would mimic ATP inhibition.

A major kinetic distinction between the C and W forms of citrate synthase is the thermal sensitivity of ATP inhibition. With the W variant, the $K_i$ of ATP is relatively insensitive to temperature up to at least 22° but rises sharply above this temperature (Fig. 8). At 35°, the $K_i$ is about 2-fold higher than at 22°. The $K_i$ for ATP of the C form increases dramatically with temperature above 15°; below 15°, the $K_i$ is insensitive to further temperature decrease, and the absolute value is very similar to the $K_i$ for the W form of the enzyme. Thus, over the normal thermal ranges for cold and warm acclimated trout, ATP modulation of liver citrate synthase is essentially independent of temperature. These changes in $K_i$ appear to parallel the changes in the $K_m$ of acetyl-CoA; since the $K_i$ of ATP may represent binding, this similarity suggests that the $K_m$ of acetyl-CoA may accurately represent an affinity constant.

Effect of pH on Trout Liver Citrate Synthases—Rahn (14) has shown that poikilotherms regulate $H^+\cdotOH^-$ ratios in extracellular fluids rather than strictly regulating the pH as is commonly observed in the homeotherm. In maintaining a constant $H^+\cdotOH^-$ ratio in the blood, an increase in blood pH occurs at
Fig. 6. Effect of ATP on the activity (V) of the C form of citrate synthase is shown on the left at various acetyl-CoA (AcSCoA) concentrations at 22°C. Double reciprocal plots of the same data are shown on the right, indicating that ATP increases the apparent $K_m$ by about 4- to 5-fold, but it does not appreciably decrease the calculated $V_{max}$. Initial assay conditions: 0.1 mM oxalacetate, 1.0 mM, 2.5 mM or 5.0 mM ATP, various concentrations of acetyl-CoA, and enzyme preparation in 0.1 M Tris-HCl buffer, pH 7.5, at 22°C.

Fig. 7. Effect of ATP on the activity (V) of the C form of citrate synthase is shown at the left at various oxalacetate concentrations at 22°C. Double reciprocal plots of the same data are shown on the right, indicating that ATP does not appreciably alter the $K_m$ of oxalacetate. Assay conditions were as in Fig. 6.

lower temperatures which parallels the increase in the ionization constant of water. Recent evidence appears to bear out Rahn's original suggestion that a similar requirement for regulation of intracellular H$^+\cdot$OH$^-$ ratio confronts the poikilotherm (15). For these reasons, an examination of the role of pH in the regulation of trout citrate synthase seemed necessary.

Although trout liver citrate synthases do not show a pH optimum between pH 6.5 and 8.5, the maximum catalytic activity increases quite strikingly as the pH is raised. Thus, the calculated $V_{max}$ at pH 8.5 for both forms of the enzyme is about 4 times higher than at pH 6.5 (Fig. 9). Similar effects of pH upon $V_{max}$ of mammalian citrate synthase have been observed (11). In the physiological pH range of blood in fishes (pH 7.3 to 8.0), the catalytic activity of the enzyme appears to be most sensitive to pH changes. The $K_m$ values of acetyl-CoA and oxalacetate do not vary between pH 7.5 and 8.5, but the $K_m$ of acetyl-CoA is decreased at pH 6.5 to two-thirds the value at pH 7.5.

An important effect of pH upon the $K_i$ of ATP is seen with citrate synthases of cold and warm acclimated fish (Fig. 10). Increasing the pH from 7.0 to 8.5 results in about a 4-fold increase in the $K_i$ of ATP. Between pH 7.5 and 8.0, the $K_i$ for the W form of the enzyme appears slightly less stable to pH change than the C form. It is clear, however, that efficiency in size of ATP modulation of both the C and W forms of citrate synthase must depend critically upon intracellular pH.

Search for Other Regulatory Metabolites—A variety of other compounds, phosphoenolpyruvate, pyruvate, citrate, malate, α-ketoglutarate, and NAD at concentrations up to 1 mM had little if any measurable effect on trout liver citrate synthase. Mg$^{2+}$ is a potent inhibitor of the enzyme. The Mg$^{2+}$ saturation
FIG. 8. Effect of temperature on the $K_i$ of ATP for liver citrate synthase of 2° and 18° acclimated trout. The $K_i$ was determined from plots of $1/V$ versus ATP concentration. ATP inhibition was assayed under conditions of 0.1 mM oxalacetate and 0.4 mM acetetyl-CoA. Other conditions were as in Fig. 6.

FIG. 9. Effect of pH on the $V_{max}$ (calculated from double reciprocal plots) for liver citrate synthases from 2° and 18° acclimated trout. Assay conditions were as given under "Methods and Materials."

FIG. 10. Effect of pH on the $K_i$ of ATP for the C form (○) and the W form (△) of trout liver citrate synthase. ATP inhibition was assayed under conditions of 0.1 mM oxalacetate and 0.4 mM acetetyl-CoA. Other conditions were as in Fig. 6.

FIG. 11. Effect of Mg$^{2+}$ on the activity (V) of liver citrate synthase from 2° acclimated fish. Assay conditions were as given under "Methods and Materials."

curve for the C form of the enzyme (Fig. 11) is hyperbolic; for the pig heart enzyme, the inhibition curve appears to be somewhat sigmoidal (13). The inhibitory effect of Mg$^{2+}$ is not due simply to increasing ionic strength, since Ca$^{2+}$ at similar concentrations does not affect the enzyme.

We did not examine Mg$^{2+}$ inhibition in any further detail, for the $K_i$ of the cation is high (about 30 mM), and large changes in Mg$^{2+}$ would appear to be required for effective modulation of the enzymes. It should be pointed out, however, that divalent cations can to some extent reduce ATP inhibition of the pig heart enzyme (13) and, in this manner, may play an important regulatory role.

FIG. 12. Effect of temperature and pH on the $K_i$ of ATP for the C form of liver citrate synthase. T, effect of temperature. The pH at any temperature is taken as the pH of blood at that temperature (14).
DISCUSSION

A number of recent studies indicate that citrate synthase is modulated by the adenylates. The enzyme from tissues of several animal species, from yeast, and from mitochondria of plants (16–19) is strongly inhibited by ATP, which causes an increase in the apparent Michaelis constant for acetyl-CoA. ADP and AMP, when tested singly, may also be inhibitory, but these inhibitors are less effective than ATP. In Escherichia coli, the situation is somewhat more complex. Citrate synthase is inhibited by ATP only at pH values above 7.5; at lower pH values, ATP acts as a positive modulator (19). The catalytic rates in the presence of AMP, however, are greater than those with ATP at all pH values examined. Moreover, in E. coli, other “products” of the Krebs citrate cycle, NADH and α-ketoglutarate (20, 21), serve as negative modulators, and a similar situation apparently prevails generally in gram-negative bacteria (22). The inhibition of citrate synthase is presumed to be of the allosteric type because the enzyme can be sensitized to its specific inhibitors without loss of enzymic activity; also, from direct electron microscope observations, it appears that citrate synthase undergoes important allosteric transitions upon binding modulators (23). Our studies are consistent with this general picture and indicate that the trout enzymes display characteristics intermediate between those of the mammalian and the bacterial enzymes.

It is critical, in considering possible physiological consequences of observed enzyme-ligand interactions, to take into account the biological context of the enzyme reaction involved. In this connection, the unique problems of metabolic control in poikilotherms living under variable and often unpredictable thermal regimes have been largely overlooked. The relationship between the apparent $K_m$ and temperature, for example, suggests that the temperature coefficient of the reaction is decreased at low substrate concentrations, and therefore that, in vivo, the $Q_10$ of the reaction will depend on cellular substrate concentrations. At low substrate concentrations, when the $K_m$ is important in determining the reaction velocity, the $Q_10$ will be low; at higher substrate concentrations, the $Q_10$ will increase. These considerations would not hold at temperatures below about 10°C, at which levels the apparent $K_m$ values appear to be less thermally sensitive. We have no information on cellular concentrations of acetyl-CoA and oxalacetate but, according to recent estimates, these may be assumed to be in the same range as, or lower than, the $K_m$ values for the trout enzymes (24, 25).

We have already noted that the $K_i$ values of ATP for both the C and W forms of citrate synthase increase with pH (Fig. 10) and with temperature (Fig. 8). Under normal biological conditions, these effects of pH and temperature may cancel each other because pH decreases with increasing temperature. This is shown for the C form of the enzyme in Fig. 12, in which the pH at any given temperature is taken as the blood pH at that temperature. A possible deduction from these data is that, in vivo, the ATP-binding site of citrate synthase is modulated by pH to maintain ATP control equally effective over a larger part of the thermal range of the species.

Another consequence of cellular pH varying with temperature relates to the pH optimum for enzyme function. Because increasing pH activates trout citrate synthase (Fig. 9), the temperature coefficient of the reaction is decreased from about 1.7 to about 1.45 when the pH is adjusted according to Rahn’s pH-temperature relationship (14). This serves as an interesting mechanism by which the effective $Q_10$ of the reaction in vivo can be decreased over a broad thermal range, irrespective of substrate concentrations. This kind of mechanism, together with the $K_m$-temperature relationship, which at low substrate levels also serves thermally to stabilize the reaction, may contribute to the low $Q_10$ values that have been reported for aerobic metabolism of liver preparations from rainbow trout (2), as well as for tissues from other poikilotherms (3, 5).

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