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Laboratory Protocol: Citrate synthase a mitochondrial marker enzyme

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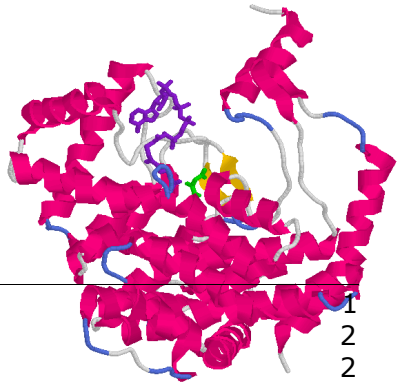

1. Background

Citrate synthase (E.c. 4.1.3.7) is a pace-maker enzyme in the Krebs cycle (citric acid cycle or tricarboxylic acid cycle, TCA). Citrate synthase, CS, has a molecular weight of 51,709 Da, with gene map locus 12q13.2-q13.3. CS is localized in the mitochondrial matrix, but is nuclear encoded, synthesized on cytoplasmic ribosomes and transported into the mitochondrial matrix. CS, therefore, is commonly used as a quantitative marker

enzyme for the content of intact mitochondria (Holloszy et al 1970; Willimas et al 1986; Hood et al 1989), although this role of CS has been questioned in developmental (Drahota et al 2004), age-related (Marin-Garcia et al 1998), physical training (Pesta et al 2011) and cardiac disease studies (Lemieux et al 2011). Proliferation of mitochondria in pathological states is sometimes associated with an increase in CS activity per cell, but CS activity in a specific tissue is frequently constant when expressed per mitochondrial protein or per mt-respiratory capacity (Renner et al 2003). Mitochondrial, cellular or tissue respiration, therefore, may be expressed per CS activity for specific applications (Kuznetsov et al 2002; Renner et al 2003; Hütter el al 2004).

1.1. Enzymatic reaction catalyzed by citrate synthase

CS catalyzes the reaction of two-carbon acetyl CoA with four-carbon oxaloacetate to form six-carbon citrate, thus regenerating coenzyme A.

Acetyl-CoA + oxalacetate + $H_2O \rightarrow$ citrate + CoA-SH (1)

1.2. Principle of spectrophotometric enzyme assay

(Srere 1969; Bergmeier 1970)

Absorbance and enzyme activity: The optical density, *OD,* of a liquid sample is related to the absorbance, *A,* by the optical path length, $[cm]^{-1}$,

$$OD = A / l = \varepsilon_B \cdot c_B \quad (2)$$

A is a dimensionless number. The path length is fixed by the dimension of the spectrophotometric cuvette. The molar extinction coefficient of the absorbing substance B, ε_B $[mM^{-1} \cdot cm^{-1}]$, is specific for the compound studied at a particular wavelength. Absorbance increases with molar concentration, c_B [mM], in the solution contained in the cuvette. The rate of increase of the absorbance is the slope, $r_A = dA/dt$, which is proportional to enzyme activity.

Citrate synthase assay: In the spectrophotometer, the rate-limiting reaction catalyzed by CS (Eq. 1) is coupled to the effectively irreversible chemical reaction (Eq. 3),

CoA-SH + DTNB $\rightarrow$ TNB + CoA-S-S-TNB (3)

The reaction product TNB (thionitrobenzoic acid) is the absorbing substance B (Eq. 2) with intense absorption at 412 nm. Therefore, the working wavelength is

412 nm. The absorbance increases linearly with time, up to 0.6-0.8 units of absorbance (over 200 s of measurement). The enzyme activity is not affected by up to 1% Triton X-100.

1.3. Temperature of enzyme assay

When CS activity is used as a marker, it is not critical to choose a physiological temperature. A constant reference temperature has to be applied for comparability of measurements. Measurements are frequently performed at room temperature, but more commonly at 30 °C (Hütter et al 2004; Kuznetsov et al 2002; Renner et al 2003; Trounce et al 1989).

2. Reagents and buffers

2.1. Prepare every month new and store at 4 °C

Tris-HCl buffer (1.0 M, pH 8.1): 2.4228 g Tris/20 ml a.d., adjust to pH 8.1 with 37% HCl (ca. 100 µl/20 ml).

Tris–HCl buffer (0.1 M, pH 7.0): 2 ml 1M Tris-HCl buffer, pH 8.1+ 15 ml a.d. Adjust pH to 7 with concentrated HCl and fill up to 20 ml with a.d.

Triethanolamine-HCl buffer (0.5 M, pH 8.0) + EDTA (5 mM): 8.06 g triethanolamine/100 ml a.d., adjust pH with 37% HCl, add 186.1 mg EDTA. pH does not change after addition of EDTA. Triethanolamine is viscous. Weigh in a beaker on the balance.

Triton X-100 (10% solution): Reagent solution is 100%, add 90 ml a.d. to 10 g (ca. 10 ml) Triton X-100.
Triton X-100 is viscous and sticky. Weigh on balance in a beaker and dissolve by stirring.

2.2. Prepare 12.2 mM acetyl-CoA, store at -20 °C

25 mg acetyl CoA + 2.5 ml a.d., make aliquots of 250 µl and store at –20 °C. Store on ice during measurement, freeze it again after the experiment.

2.3. Prepare fresh every day

Triethanolamine-HCl-buffer (0.1 M, pH 8.0): 1 ml of 0.5 M triethanolamine-HCl-buffer of pH 8.0 + 4 ml a.d.

Oxalacetate (10 mM, pH 8.0): 6.6 mg oxalacetate + 5 ml of 0.1 M triethanolamine-HCl-buffer of pH 8.0.

DTNB (1.01 mM, pH 8.1): 2 mg DTNB + 5 ml of 1 M Tris-HCl-buffer of pH 8.1.

2.4. Chemicals

Name	FW	Stock solution	Company, product code, storage	Comments
Tris(hydroxymethyl) aminomethane $C_4H_{11}NO_3$	121.14	1.0 M; 2.4228 g/20 ml a.d.	Sigma, 252859 store at RT	pH 8.1 with HCl, to obtain Tris-HCl buffer.
Triethanolamine $C_6H_{15}NO_3$	149.19	0.5 M; 8.06 g/100 ml a.d.	Sigma, 90279 store at RT	pH 8.0, viscous liquid; harmful.
EDTA (Ethylenediaminetetra acetic acid disodium salt dehydrate) $C_{10}H_{14}N_2O_8Na_2{\cdot}2H_2O$	372.20	5 mM; 186.1 mg/100 ml of 0.5 M triethanolamine-HCl buffer pH 8.0	Sigma, E1644 store at RT	Chelator for heavy metals, added to avoid interference with SH-groups.
Triton X-100, $C_{34}H_{62}O_{11}$	646.87	10%; 10 g/100 ml a.d.	Sigma, T8532 store at 4 °C.	Viscous liquid; detergent.
Oxalacetic acid $C_4H_4O_5$	132.07	10 mM; 6.6 mg/5 ml of triethanolamine-HCl-buffer	Sigma, O4126 store at -20 °C.	Irritant.
DTNB (5,5'-Dithiobis(2-nitrobenzoic acid), Ellman's reagent, $C_{14}H_8N_2O_8S_2$	396.35	1.01 mM; 2 mg/5 ml of Tris-HCl-buffer	Sigma, D8130 store at RT	TNB-S-S-TNB (di-thionitrobenzoic acid); irritant.
Acetyl CoA (acetyl coenzyme A), lithium salt, $C_{23}H_{38}N_7O_{17}P_3SLi$	816.50	12.2 mM; 25 mg/2.5 ml a.d.	Sigma, A2181 store at -20 °C.	Prepared enzymatically, toxic.
Citrate synthase, CS		8.6 mg prot./ml	Sigma, C3260 store at 2-8 °C.	From pig heart; harmful; crystalline suspension in 2.2 M $(NH_4)_2SO_4$, pH 7. Specific activity: 200 IU/mg protein at 37 °C, varies with Lot Number.

3. Sample preparation

Freeze sample in liquid nitrogen and store frozen at -80 °C. Alternatively, a short storage (within 1 month) at −20 °C is possible. During measurements, store the sample on ice. CS activity of cells is stable during storage (a few hours) on ice.

Permeabilized fibres and whole tissue slices have to be homogenated with an Ultra Turrax (T10 basic; IKA) for 20 to 30 s at level 4 before CS measurement.

3.1. Isolated mitochondria

Due to very high CS activity of isolated mitochondria, the suspension for measurement can be prepared by dilution (1:3 to 1:10) of a frozen stock mitochondrial suspension (-80 °C; usually ca. 50 mg of mitochondrial protein per ml). Immediately after thawing, add 20 µl of mitochondrial suspension to 180 µl of 0.1 M Tris-HCl buffer, pH 7.0 (RT). During measurement store on ice. Freeze stock suspension again. 10-25 µl mitochondrial suspension (5 mg/ml) is used for each spectrophotometric measurement.

3.2. Suspended cells

For typical cells (HUVEC, lymphocytes) at $1\text{-}2\cdot10^6$ cells/ml, take replicates of 110 µl samples into Eppendorf tubes, freeze in liquid nitrogen, and store until measurement.

3.3. Samples from the Oroboros O2k

After the experiment collect everything from the chamber with a pipette and rinse/wash the chamber with 2 ml of fresh medium. Collect the whole sample in a 15 ml Falcon, freeze in liquid nitrogen and store at -80 °C until measurement.

3.4. CS Standard

Preparation: As a standard, commercial citrate synthase is diluted 1:500 in 0.1 M Tris-HCl buffer, pH 7.0 (RT). Accurate dilution is critical and is achieved by adding 2 µl of CS standard (using a 10 µl Hamilton syringe) to 998 µl of buffer. Starting with a protein concentration of 8.6 $mg\cdot cm^{-3}$ in the undiluted CS standard, this yields a final protein concentration of 0.0172 $mg\cdot cm^{-3}$ in the sample, of which 5 µl are added to a volume of 995 µl of reaction mix (1:200). At the end, the dilution is 1:100.000. Always use freshly diluted enzyme.

Application: The CS standard serves as a check of chemicals and assay conditions, and may even be used for final correction of results. A standard (at least in replicate) is included at each day of measurement, if a large number of samples are processed collectively.

Different CS standards: The storage-age of the standard has to be checked critically. If a new CS standard is applied, the Lot Number is noted, together with the specific activity and the protein concentration provided by the supplier. For instance, a general value of 200 IU/mg protein is listed in the Sigma catalogue, whereas a specific

activity of 215 IU/mg (8.6 mg/ml) is given for Lot Number 121H9500 (37 °C).

4. Measurement: Spectrophotometer HP8452A Diode Array

4.1. Measurement of CS Activity in 1 ml Cuvette

The spectrophotometer has to be switched on about 10 min before measurement. First, before turning on the photometer, remove all cuvettes from the cuvette holder, then switch on the power supply of the spectrophotometer, the computer, and the monitor. Next, set up a kinetic program at 412 nm:
Program → **HP8452.BAT** on drive **C:**
Measurement → ***kinetics***
Choose parameter file: Files → Recall Parameter File

- → **CITSYN01.mkp** (1 sample at position 1 per measurement)
- → **CITSYN02.mkp** (3 samples in parallel at positions 1, 2, and 3 per measurement)
  - Number of samples = 3
  - Interval time = 10 s
  - Total time = 120 s
  - Read average time = 0.5 s

4.2. Blank measurement

Add 1 ml a.d. into a glass cuvette and insert the cuvette into the spectrophotometer at position 1

- → **Scan Screen**: an empty plot appears
- → **Pre run [Blank]**
- → **Measure Blank** – the value will be saved automatically and graph (an example is given in Fig. 1) will be displayed.
- → **Press ESC or right mouse button** to remove the open box in front of the graph.

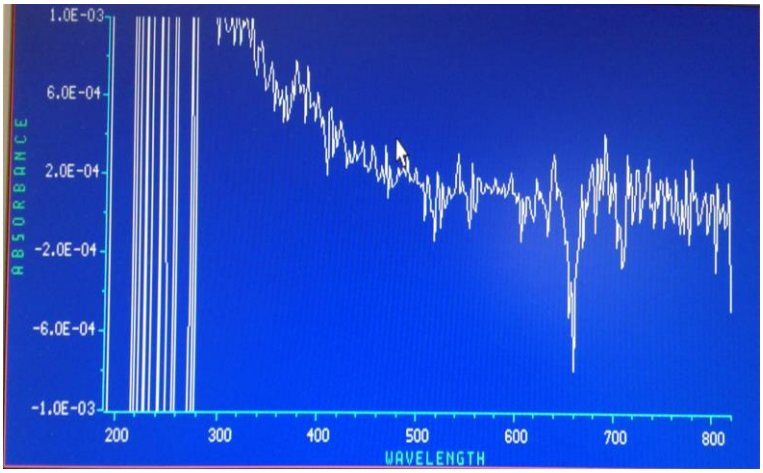

Fig. 1: Display of the curve of the blank measurement.

4.3. Sample measurement

- ➔ **Start Run:** for new file
- ➔ **Data name [e.g. AW001] --> *enter***
- ➔ **Comment [sample numbers] --> *enter***
- ➔ **F1 for start:** The linear increase of absorbance is measured over approximately 200 s.

Write into the protocol the measured rate of absorbance change, r_A = dA/dt, in AU/s (see Section 5). Also check the graph of the measured sample for linearity by going back with **ESC**, with left mouse button on **RESCALE**, then double click on **ZOOM OUT** – the graph (example in Fig. 2) is displayed.

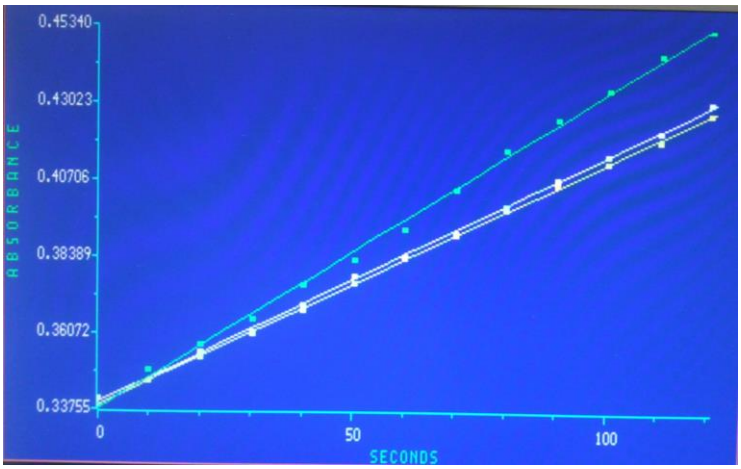

Fig. 2: Graph displaying the simultaneous measurement of 3 samples.

4.4. Experimental procedure

Add all reaction components (except for the 10 mM oxalacetate solution) in the given sequence (like this the components are gently mixed and the reaction mixture turns yellow):

	Component	V added (µl)	Final conc.
1	10 % Triton X-100	25 µl	~ 0.25 %
2	Acetyl CoA	25 µl	~ 0.31 mM
3	1.01 mM DTNB	100 µl	~ 0.1 mM
4	V_{sample} *	See table below	~ 5 mg/ml
5	H_2O_{dest}	(800 µl - V_{sample})	
6	Oxalacetate	50 µl	~ 0.5 mM

Prepare all components in a glass cuvette except oxaloacetate. If 3 samples are measured at one time, prepare 3 cuvettes with components. Add oxaloacetate into first cuvette, seal with parafilm and your thumb. Swivel gently 3 times. Remove parafilm and place cuvette at position 1 (front). If 3 samples are measured, prepare them consecutively. Immediately

after the 3rd sample start measuring with F1 or clicking on 'Start'.

The first 3 samples measured should be:

- MiR06 (10 µl)
- CS standard (5 µl)
- CS standard replicate (5 µl)

Amount of sample (µl) necessary for CS measurements:

A protein concentration of 5 mg/ml is optimal for CS activity measurements. The following dilutions and sample additions were applied:

Sample	Dilution	V_{sample} [µl]
CS standard (see Section 3)	-	5
Medium (MiR06/MiR07)	-	10
Cell suspension	n.d.	100
Heart homogenate	n.d.	5
Crude liver homogenate	1:4	10
Crude brain homogenate (Hmt)	1:4	5
1 spin homogenate (Smt)	1:1	10
Isolated brain mitochondria (Imt)	1:3	10

5. Data analysis: Calculation of specific CS activity

5.1. Absorbance, concentration and rate of reaction

The rate of concentration change of the absorbing compound B in the cuvette, dc_B/dt, is calculated from the rate of the absorption change (Eq. 2),

$$dc_B/dt = \frac{dA/dt}{l \cdot \varepsilon_B} = \frac{r_A}{l \cdot \varepsilon_B} \quad (4)$$

The reaction flux per unit volume, J_V, in the cuvette is,

$$J_V = dc_B/dt \cdot \nu_B^{-1} \quad (5)$$

where ν_B is the stoichiometric number of compound B (Gnaiger 1993), which is equal to 1 in the coupled reactions (1) and (3).

5.2. Specific enzyme activity: reaction rate per unit sample

The specific enzyme activity is proportional to the experimental reaction flux (Eq. 5) and inversely proportional to the dilution factor, $V_{sample}/V_{cuvette}$ and to the mass concentration, ρ [mg·cm^{-3}] or cell density [10^6·cm^{-3}] in the sample, V_{sample}. The specific enzyme activity, ν, is the velocity of the enzyme-catalyzed step per unit sample, measured under experimental incubation conditions with saturating substrate. Combining Eq. 4 and 5,

$$\text{Specific activity}: \quad v = \frac{r_A}{l \cdot \varepsilon_B \cdot \nu_B} \cdot \frac{V_{cuvette}}{V_{sample} \cdot \rho} \qquad (6)$$

v Specific activity of the enzyme is expressed per mg protein or per million cells [IU/mg protein or IU/10^6 cells], depending on ρ. Enzyme activity is frequently expressed in international units, IU [µmol/min]. 1 IU of CS forms 1 µmol of citrate per min. Note that the minute is used here as the unit of time (although the second is the *SI* base unit; Gnaiger 1993).

$r_A = dA/dt$ Rate of absorbance change [min^{-1}] (Eq. 4).

l Optical path length (= 1 cm).

ε_B Extinction coefficient of B (TNB) at 412 nm and pH 8.1 = 13.6 $mM^{-1} \cdot cm^{-1}$ = (13.6 $mmol \cdot dm^{-3})^{-1} \cdot cm^{-1}$.

ν_B Stoichiometric number of B (TNB) in the reaction (Eq. 3) (= 1).

$V_{cuvette}$ Volume of solution in the cuvette (= 1000 µl).

V_{sample} Volume of sample added to cuvette (5 - 100 µl).

ρ Mass concentration or density of biological material in the sample, V_{sample} (protein concentration: $mg \cdot cm^{-3}$; cell density: $10^6 \cdot cm^{-3}$).

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7. Author contributions

This MiPNet Protocol was originally prepared by Kuznetsov AV, Lassing B, Gnaiger E as MiPNet08.14 in 2003 and further edited by Wiethüchter A in 2011. The newest version as MiPNet17.04 was edited by Eigentler A, Draxl A, Gnaiger E in February-May 2012.

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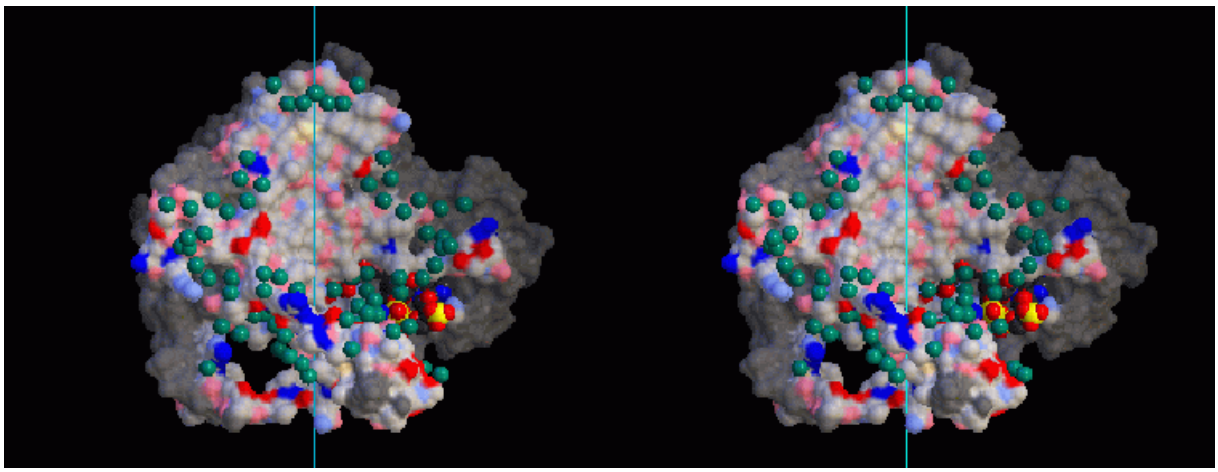
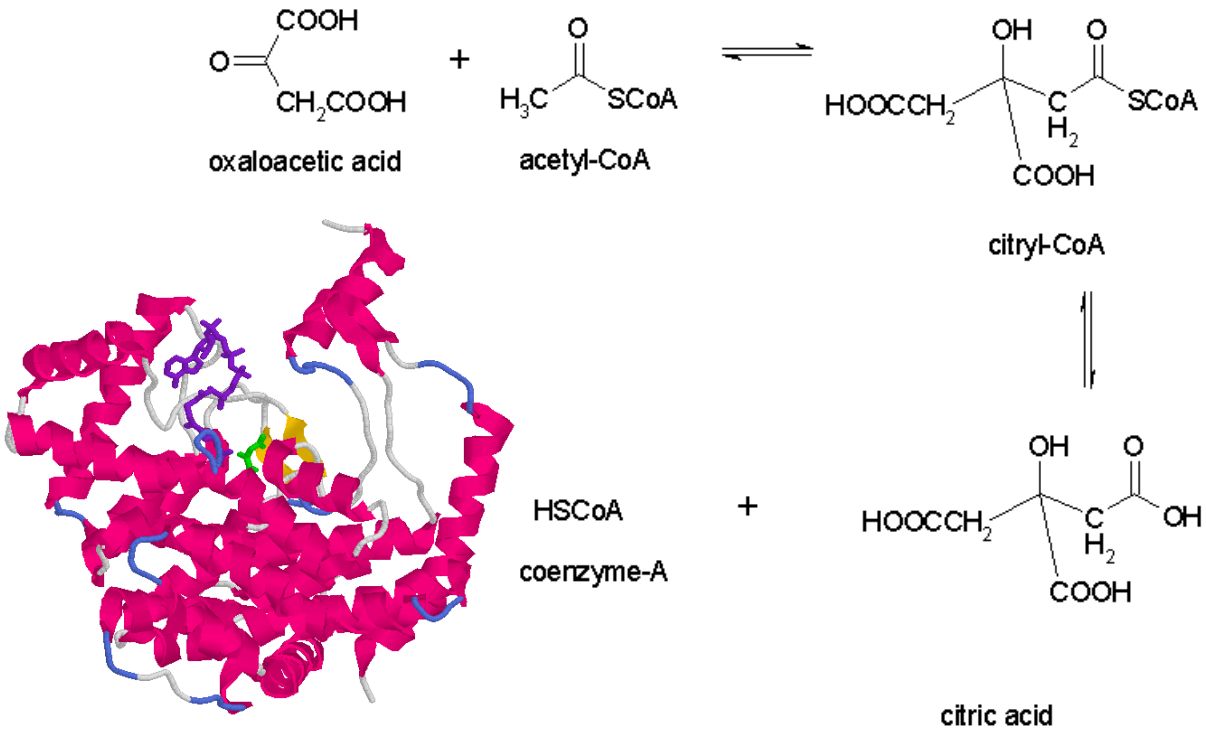
Appendix



Cross-eyed stereo image of citrate synthase
www.scripps.edu/pub/goodsell/interface/interface_images/1csh.html



Citrate synthase complexed with oxaloacetate (green) and the CoASH analog amidocarboxymethyldethia coenzyme A (purple).
http://chemistry.gsu.edu/Glactone/PDB/Proteins/Krebs/1csh.html