Cobalamin-Dependent Methionine Synthase Assay

This protocol is based on the method developed by the Matthews lab [Drummond, J. T., Jarrett, J., Gonzalez, J. C., Huang, S., and Matthews, R. G. (1995) *Anal. Biochem.* **228,** 323-329; Jarrett, J. T., Goulding, C. W., Fluhr, K., Huang, S., and Matthews, R. G. (1997) *Methods Enzymol* **281,** 196-213]

The enzyme catalyzes the following reaction:

$$5\text{-}CH_3\text{-THF} + \text{L-homocysteine} \rightarrow \text{THF} + \text{methionine}$$

The product, THF, is detected spectrophotometrically following its conversion to 5,10-methenyl-THF by heating with formate in acid:

$$\text{THF} + \text{formate} \rightarrow CH^+\text{-THF} + 2\ H_2O$$

At acidic pH, CH^+-THF has an extinction coefficient of 26,500 $M^{-1}cm^{-1}$ at 350 nm

1. Add the following to 12x75mm glass tubes:

494 µL	H_2O
80 µL	1.0 M KPO_4 (pH 7.2)
40 µL	500 mM DTT
4 µL	3.8 mM AdoMet
4 µL	100 mM L-homocysteine
50 µL	enzyme sample
80 µL	500 µM hydroxocobalamin

mix well, then preincubate at 37°C for 5 minutes

2. Initiate reactions with 48 µL 4.2 mM CH_3THF

   mix well, incubate at 37°C for 10 minutes.

3. Stop reactions with 200 µL 5N HCl/60% formic acid
   mix well, then incubate at 80° C for 10 minutes in a heat block. Cool to room temperature.

4. Transfer each 1 ml reaction to 1.5 mL microcentrifuge tube and centrifuge at room temperature at top speed for 5 minutes to pellet precipitated protein, then measure absorbance at 350 nm (zero the instrument against water). This centrifugation step is not necessary for samples with low protein concentration.

Blanks -- two kinds of blanks can be used:

(i) The simplest is a "no enzyme" blank. Use 50 µl of whatever buffer your sample was in. This is fine for monitoring column fractions or when all the samples have the same protein content/concentration (e.g. kinetics). This single blank can be subtracted from all of the

samples in the assay. The no enzyme blank is typically 0.280 – 0.300 A_{350} in our hands.

(ii) "minus homocysteine" blank. For every enzyme sample, a second set of tubes contains the same amount of enzyme, but minus homocysteine. Use 4 µl of H_2O instead. This blank is necessary when measuring activity in crude extracts.

All samples and blanks should be performed in duplicate or triplicate. A standard curve for THF can be prepared under a given set of assay conditions to correct for variations in yield and linear range. For following activity in column fractions, etc., a standard curve is not necessary.

Calculations

Activity in nmol/min (milliunits, mU) is calculated from the extinction coefficient of CH^+-THF in acid = 26.5 X 10^{-6} nM^{-1}, the volume of the reaction, and the time:

$$Corrected\ A_{350} \times \left(\frac{1.0\ ml}{26.5 \times 10^{-6} nM^{-1} \times 10\ \text{min} \times 10^{3} ml/L} \right) =$$

$$Corrected\ A_{350} \times 3.774$$

Reagents

1. L-homocysteine
   Dissolve 50 mg L-homocysteine thiolactone (Sigma) in 1.7 ml H_2O. Add 0.83 ml 0.8 M NaOH, bubble with argon for 5 min and incubate at 45°C, 6 min. Acidify with 5.0 M acetic acid to pH 5 (check with pH paper); dilute to 3.3 ml with argon-bubbled H_2O to yield ~ 100 mM solution. Store in 1 ml aliquots at –80°C.

   Check actual concentration by titration with DTNB (see pp. 155 and 220 of Chemical Modification of Proteins, by Means and Feeny, Holden-Day, Inc., 1971):
   •prepare 10 mM DTNB stock in 50 mM K•Phos (7.0) (39.6 mg/10 ml)
   •dilute 100 mM hcy stock 1:100 and add 50 µl to 900 µl 0.1 M Na•Phos (8.0)
   •add 50 µl DTNB reagent. Stand 2-3 min, read at 412 nm.
   •subtract buffer blank, divide corrected A_{412} by the extinction coefficient for the liberated thionitrobenzoate anion (13.6 mM^{-1}) to calculate actual L-homocysteine concentration in stock

2. (6*R,S*)5-methyl-THF (monoglutamate form; calcium salt, Schircks Laboratories, Jona, Switzerland): prepare 4.2 mM stock in 8 mM Na•Ascorbate. Store in dark under argon at –20°C

3. hydroxocobalamin (Sigma H-8017 or ICN 157408): dissolve to 500 µM in H_2O. Store in dark at 4°C.

4. *S*-adenosyl-L-methionine (chloride salt, Sigma or *p*-toluensulfonate salt, BASF): dissolve to 3.8 mM in 1 mM HCl. Store at –70°C.

5. formate/HCl: slowly add 41.6 ml concentrated HCl (12 N) to 58.4 ml of 88% formic acid.