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ULTRAMICRO ANALYSIS OF ENZYMES AND SUBSTRATES BY ENZYMATIC AMPLIFICATION REACTIONS " *ENZYMATIC CYCLING* "

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Abstract-The principle and kinetics of enzymatic cycling are described. The enzymatic cycling reaction is catalyzed by a couple of enzymes and amplifies stoichiometrically a pair of substrates participating in the reaction. Many biological substances can be converted quantitatively to substrates that can be amplified and measured by cycling reactions. Three cyclings in routine use (NAD, NADP and CoA cyclings) are summarized and compared. Each cycling provides an amplification of more than 10,000 times per hour and the combination of these cyclings (double and triple cyclings) results in an even larger gain in sensitivity. Theoretically single molecules can be measured by using the triple cycling. As examples, the determination of ATP and acetylcholine synthesizing enzyme (choline acetyltransferase, EC 2.3.1.6) are described. A novel method (*popcorn ball technique*) discriminating CoASH and acetyl-CoA, which are the substrates amplified by CoA cycling, is explained in detail. The cycling reactions are believed to be capable of replacing many radiometric methods.

INTRODUCTION

The " *enzymatic cycling* " is a microdetermination method to measure less than 10^{-15} moles of biological substances, by means of coupled enzymatic reactions. Originally this method was developed in " Neurochemistry " (biochemistry of central and periferal nervous system). The central nervous system is composed of nerve cells(neurones), three kinds of glial cells, and endotherial cells in capillary nets. These components are intermingled and form the complicated structure of the central nervous system. Because of this complicated structure, the analysis of a mass of nervous tissue does not provide a clear view of biochemical characteristics belonging to each kind of cell, especially neurones. To obtain the clear view, an analysis of a single cell has been undertaken by many workers, who have made efforts in developing several microdetermination methods. The enzymatic cycling is one of these methods, but is interesting in general since this method is applicable for analyzing even single molecules of biological substances and enzymes.

ENZYMATIC CYCLING

CoA cycling (Fig. 1, Ref. 1), one of the enzymatic cyclings available at present, is taken as an example to explain the principle and kinetics of enzymatic cycling reactions.

Principle

In the presence of acetyl-P and oxalacetic acid in excess, CoASH is acetylated by PTA to acetyl-CoA with production of an amount of P_i equal to that of CoASH. Acetyl-CoA is subsequently deacylated by CS and returns to CoASH, producing citrate in the same amount as that of original CoASH. One thousand reactions in cyclic fashion accumulate P_i and citrate in an amount exactly (stoichiometrically) one thousand times that of original CoASH.

Citrate is converted quantitatively to α-ketoglutarate by the following two enzyme reactions in the presence of excessive $NADP^+$, and the fluorescence of NADPH is determined.

citrate -(aconitase)→ *cis*-aconitate -(aconitase)→ isocitrate

isocitrate + $NADP^+$ -(isocitrate dehydrogenase)→ α-ketoglutarate + CO_2 + NADPH + H^+

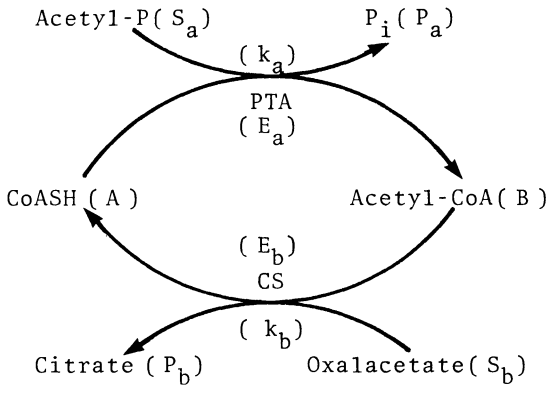


Fig. 1. Principle of CoA cycling. PTA, phosphotransacetylase; CS, citrate synthase. Symbols in parentheses are: S, substrate; E, enzyme; P, product; k, first-order reaction rate constant ; A and B, the substrates to be amplified in the cycling reaction.

This reaction is called " *Indicator Reaction* ".

Kinetics

The symbols, given in the parentheses in Fig.1, are used to explain the kinetics of cycling reactions. In the cycling reaction, the following three conditions must be met: 1) the concentrations of S_a and S_b are higher than the Michaelis constants (K_m's) of the enzymes E_a and E_b for these substrates; 2) the concentrations of A and B are considerably lower than the K_m's of E_a and E_b for these substrates; 3)the concentrations of E_a and E_b are sufficiently low. Under these conditions both reactions are of the first order with respect to A and B; the first-order reaction rate constants are given as K_a and K_b:

$$dA/dt = -k_aA + k_bB \quad (1)$$

$$dB/dt = k_aA - k_bB \quad (2)$$

The solution of these differential equations is given by equations (3) and (4) wherein (3) A_0 and B_0 are the concentrations of A and B at t = 0, and C = A + B (constant).

$$A = [k_b/(k_a + k_b)]C + [(k_aA_0 - k_bB_0)/(k_a + k_b)]\, e^{-(k_a + k_b)t} \quad (3)$$

$$B = [k_a/(k_a + k_b)]C - [(k_aA_0 - k_bB_0)/(k_a + k_b)]\, e^{-(k_a + k_b)t} \quad (4)$$

The values of k_a and k_b are more than 10 min^{-1} under the experimental conditions for the cycling reactions and $e^{-(k_a + k_b)t}$ approaches 0 rapidly after the reaction starts. Then A and B become constant and equal the first terms in the above equations immediately after the reaction starts (steady state). The ratio of A to B in the cycling reagent equals the ratio of k_b to k_a in the steady state. The concentrations of products are given as follows:

$$P_a = \int_0^t k_aA\,dt = [k_ak_b/(k_a + k_b)]Ct, \qquad P_b = \int_0^t k_bB\,dt = [k_ak_b/(k_a + k_b)]Ct \quad (5)$$

These equations indicate that the cycling reaction amplifies A and B to the same extent and that the amounts of products are the same and proportional to the sum of the concentrations of both substrates. The amount of product is also proportional to the reaction time. The coefficient k_c as in expressed equation (6) is the cycling rate or amplification rate.

$$k_c = k_ak_b/(k_a + k_b) \quad (6)$$

Under the three conditions as above, k_a and k_b are in proportion to the concentrations of E_a and E_b respectively. Therefore the cycling rate k_c is proportional to the concentrations of both cycling enzymes. However the enzymes bind the substrates A and B to catalyze the reaction and the concentrations of free substrates diminish when the enzyme concentrations increase. This binding reduces the cycling rate at the higher concentrations of enzymes and the cycling rate levels off finally. As a result, each cycling reaction has its own maximum cycling rate (see Table 1). The kinetics at the higher concentrations of enzymes were described in detail by S.Cha and C.-J.M.Cha (Ref. 2).

TABLE 1. Enzymatic cyclings

	A§	B§	E_a/E_b§	S_a/S_b§	P_a/P_b§	k_{cmax} (cycles/hr)	Reference
NAD cycling	NAD^+	NADH	alcohol dehydrogenase / malate dehydrogenase	ethanol / oxalacetate	acetaldehyde / malate*	60,000	(3) Kato et al.
NADP cycling	$NADP^+$	NADPH	glucose-6-P dehydrogenase / glutamate dehydrogenase	glucose-6-P / α-ketoglutarate	6-P-gluconate** / glutamate	20,000	(4) Lowry et al.
CoA cycling	CoASH	Acetyl-CoA	phosphotransacetylase / citrate synthase	acetyl-P / oxalacetate	phosphoric acid / citrate***	37,500	(1) Kato

§ Symbols given in Fig. 1.; k_{cmax}, maximum cycling rate
The products are determined as follows in the indicator reactions
*malate + NAD^+-(malate dehydrogenase)→ oxalacetate + NADH + H^+; oxalacetate + hydrazine → oxalacetate hydrazone (to eliminate oxalacetate)
or malate + $NADP^+$-(malic enzyme)→ Pyr + CO_2 + NADPH + H^+
**6-P-gluconate + $NADP^+$-(6-P-gluconate dehydrogenase)→ ribulose-5-P + CO_2 + NADPH + H^+, ***For citrate, see the text.

Kinds of cycling reactions

Three kinds of cycling reactions, which are carried out routinely in the author's laboratory, are listed in Table 1. In NAD and NADP cyclings, the oxidoreduction of both nucleotides is catalyzed by cycling enzymes. The combination of these cyclings makes it possible to obtain amplification in excess of the maximum cycling rate of each cycling. When NADPH formed in the indicator reaction for CoA cycling, for instance, is subsequently amplified by NADP cycling (CoA-NADP double cycling), 7.5×10^8 (= 37,500 x 20,000) fold amplification per hr is maximally obtainable. Similarly NAD-NAD, NAD-NADP and NADP-NADP double cyclings have been utilized to determine 10^{-16} moles of substrate in single neurones (Ref. 5 and 6). If NADPH, formed in the indicator reaction after CoA-NADP double cycling, is amplified again by NADP cycling (CoA-NADP-NADP triple cycling), 1.5×10^{13} fold amplification per hr can be obtained. The sensitivity of a fluorometer for measuring NADPH and NADH is enough to determine 10^{-10} moles of these substrates. Therefore 10^{-23} moles of substrates can be amplified and determined by the triple cycling. As the mole number of a single molecule is 0.15×10^{-23} (1/N; N = 6.023×10^{23}, Avogadro number), even one molecule of substrate is measurable by using triple cycling. Thus sensitivity of the cycling reaction system is theoretically limitless.

The three cyclings listed in Table 1 are avialable to determine a vast number of biological substances and enzymes as described below. In addition to these cyclings, adenylate and guanylate cyclings (Ref. 7 and 8) were devised and used to amplify and measure cyclic AMP and cyclic GMP respectively. These cyclings cannot be applied to determine other substances.

DISCRIMINATING MEASUREMENT OF SUBSTRATES

One of the substrates A and B of the three cyclings in Table 1 can be easily eliminated with preservation of the other substrate prior to the cycling reactions. This discrimination makes it possible to measure each substrate separately and results in expanding the application range of these cyclings.

$NAD(P)^+$ and NAD(P)H

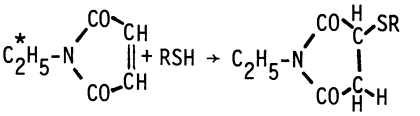
The oxidized form is stable in the acidic solution (pH ~1), but the reduced form is destroyed instantly at the acidic pH. In contrast, the reduced form is fairly stable in the alkaline solution (pH 13~14), and the oxidized form is very unstable under this condition (Ref. 9). In order to eliminate $NAD(P)^+$ from the mixture of both forms, the pH of the mixture is adjusted to 14 by adding sodium hydroxide. The mixture is then heated for 15 min at 60°C. NAD(P)H can be easily eliminated by adjusting the pH of the mixture to pH 1 with hydrochloric acid. The desired form is preserved almost completely in both cases.

CoASH and acetyl-CoA

From the mixture of CoASH and acetyl-CoA, CoASH is eliminated by forming thiol ether with NEM (N-ethylmaleimide, Fig. 2, A). Two moles of NEM per mole of CoASH is added to the mixture and allowed to react for 20 min at 30°C. The remaining NEM is eliminated similarly with GSH (reduced glutathione), which is added into the CoA cycling reagent (Ref. 1). In order to eliminate acetyl-CoA from the mixture, a new method was devised and will be explained in detail. The mixture is added to Sepharose-(glutathione-2-pyridl disulphide) conjugate (Activated-Thiol Sepharose 4B, Pharmacia, Uppsala, Sweden) and CoASH forms disulphide with this conjugate (Fig. 2, B, Thiol-disulphide interchange reaction). This interchange reaction liberates thiopyridone. The disulphide formed from the Sepharose conjugate and CoASH can be washed with buffer to eliminate acetyl-CoA and thiopyridone. In the next step, CoASH is liberated by adding the high concentration of DTT (dithiothreitol); CoASH is amplified and measured by CoA cycling. The Sepharose conjugate was used originally to purify -SH enzyme, Papain, and the purification procedure was called "covalent chromatography" (Ref. 10). The Sepharose conjugate is washed thoroughly and swollen in 5 mM Tris-HCl,pH 7.5 and is stored at 4°C. The Sepharose beads contain about 3.5×10^{-6} moles of disulphide/g of dry weight and are suspended at the concentration of 50 mg of dry weight/ml of the above buffer. A micropipette with a flat tip is plugged loosely at the constriction with nylon fiber (Nylon fiber filter paper, No.1150 with pore size of 15 - 40 μm, Toyo Roshi, Tokyo, Japan). This plug prevents Sepharose from passing through and forms a small column when the Sepharose suspension is sucked into the micropipette (Fig. 3). All the following procedures are performed under the microscope. The Sepharose column is puffed into an " oil well " filled with mineral oil. The oil well is a reaction vessel, which is a small well (2 x 2 mm) drilled in a teflon plate (30 x 150 mm). This was devised to carry out reactions in a small volume at the order of μl to *nl* under the microscope and prevent evaporation of the reagent droplet placed at the bottom of the oil well by covering it with mineral oil. From the small mass of Sepharose beads,the suspension medium is completely sucked up with a micropipette having a sharp tip. The Sepharose mass looks like a " *popcorn ball* " at this step. Therefore, this elimination method of acetyl-CoA was named *"popcorn ball technique"*.

A) Addition of Thiol to -C=C-:

(CoASH + Acetyl-CoA) + NEM* →
Acetyl-CoA + CoAS-NEM



B) Thiol-Disulphide Interchange:

1.(CoASH + Acetyl-CoA)+ Sph-GS-SPy*
→ Sph-GS-SCoA + 2-Thiopyridone#

2.Sph-GS-SCoA + Dithiothreitol →
CoASH + Sph-GS-DTT

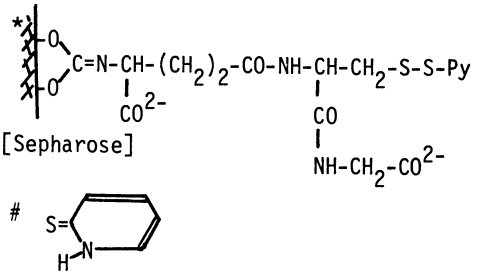


Fig. 2. Elimination reactions for CoASH and acetyl-CoA. Details are given in the text.

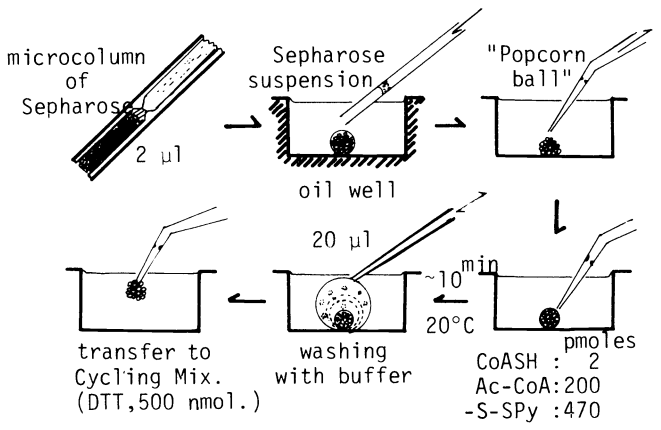


Fig. 3. Elimination procedures of acetyl-CoA (*Popcorn ball technique*). For detail, see the text.

To the popcorn ball, the mixture of CoASH and acetyl-CoA is added and allowed to stand for 10 min at 20°C. Examples of suitable amounts of CoASH, acetyl-CoA and disulphide are given in Fig.3. After all CoASH forms disulphide with the Sepharose conjugate, the popcorn ball is washed five times with times volume of Tris-HCl, pH 7.5 as much as that of the Sepharose beads. The washing buffer is sucked up completely and the popcorn ball is made to stick to the tip of

a micropipette under light suction made with the mouth from the other orifice of the pipette. The Sepharose beads are in this way transfered into the cycling reagent (50 µl) containing 10 mM DTT and the cycling reaction is carried out to amplify the liberated CoASH.

APPLICATION FOR ASSAYING BIOLOGICAL SUBSTANCES

Because of the discriminating methods for the coupled substrates in NAD, NADP and CoA cycling reactions, these cyclings have a wide range of application.
NADP cycling can be utilized to determine a small amount of ATP, which is converted to an equal amount of NADPH by using the following series of reactions.

$$\text{ATP} + \text{glucose} \xrightarrow{(\text{hexokinase})} \text{glucose-6-P} + \text{ADP}$$

$$\text{glucose-6-P} + \text{NADP}^+ \xrightarrow{(\text{glucose-6-P dehydrogenase})} \text{6-P-gluconate} + \text{NADPH} + \text{H}^+$$

$NADP^+$ and glucose are added to the reagent in excess and hexokinase and glucose-6-P dehydrogenase are used as auxiliary enzymes. After $NADP^+$ is eliminated as described above, NADPH formed is amplified and measured by NADP cycling. These reactions are called "*Conversion Reactions*". When excessive glucose, ATP and $NADP^+$ are added with glucose-6-P dehydrogenase in the conversion reaction reagent, the hexokinase activity in impure enzyme sources like tissues can be determined.
As an example to utilize CoA cycling, the determination of choline acetyltransferase (EC 2.3.1.6) is explained.

$$\text{choline} + \text{acetyl-CoA} \xrightarrow{(\text{choline acetyltransferase})} \text{acetylcholine} + \text{CoASH}$$

In the presence of choline and acetyl-CoA in excess, choline acetyltransferase in tissue produces acetylcholine and CoASH in equal amounts. Acetyl-CoA is eliminated as above and CoASH formed is amplified and assayed by using CoA cycling (details will be given elsewhere). In previous assay methods, ^{14}C or ^{3}H-labelled acetyl-CoA has been used as substrate and the product, labelled acetylcholine, had to be separated chromatographically or by selective precipitation prior to the radiometric assay. But in the present assay, the preliminary separation of CoASH is unnecessary because of the selective amplification of the small amount of the product.
All biological substances which are intermediates, or catalysts for metabolic pathways, in which NAD, NADP, CoASH and acetyl-CoA are involved as coenzymes can be determined by cycling reactions.

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