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Enzymatic cycling method using creatine kinase to measure creatine by real-time detection

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ABSTRACT

We have developed a novel enzymatic cycling method that uses creatine kinase (CK) to measure creatine. The method takes advantage of the reversibility of the CK reaction in which the forward (creatine phosphate forming) and reverse reactions are catalyzed in the presence of an excess amount of ATP and IDP, respectively. Real-time detection was accomplished using ADP-dependent glucokinase (ADP–GK) together with glucose-6-phosphate dehydrogenase. ADP, one of the cycling reaction products, was distinguished from IDP by using the nucleotide selectivity of the ADP–GK. The increasing level of ADP was measured from the level of reduced NADP at 340 nm. The method is appropriate for an assay that requires high sensitivity because the rate of increase in absorbance at 340 nm is proportional to the amount of CK present in the reaction mix. We reasoned that the method with CK in combination with creatinine amidohydrolase could be used to assay creatinine, an important marker of kidney function. Our results confirmed the quantitative capability of the assay.

Creatine kinase (CK) is a crucial enzyme found in many vertebrate tissues, where it contributes to the maintenance of homeostasis by catalyzing the reversible interconversion of creatine and the high-energy phosphate compound creatine phosphate (CP) [1–3]. CK in blood is often derived from internal organs and can be an indicator of pathology, which makes CK a useful clinical marker for the diagnosis of a range of illnesses [4]. Assays to measure creatine and creatinine using CK and creatinine amidohydrolase in combination with pyruvate kinase (PK) and lactate dehydrogenase (LDH) have already been developed [5,6].

Enzymatic cycling is a highly sensitive method that is often used in analytical chemistry. However, the application of enzymatic cycling in the field of clinical chemistry has been hindered by its relative complexity such as the need for multi-operational steps [7,8]. One example of the practical application of enzymatic substrate cycling is in the measurement of bile acids using 3α-hydroxysteroid dehydrogenase (3α-HSD) and carnitine with carnitine dehydrogenase. Here, the reversibility of dehydrogenase enzymes is exploited in the presence of an excess amount of both reduced NAD and thio-NAD^+, an analog of NAD^+, where the increasing rate of reduced thio-NAD formation can be measured directly at 400 nm [9–11].

The purpose of the current study was to investigate whether kinases follow the same principles of substrate cycling as dehydrogenases for applications in clinical laboratory testing. To this end, CK was chosen as a model for studying a reversible kinase reaction. In addition, rabbit skeletal muscle CK can use various nucleoside phosphates, including deoxyadenosine diphosphate (dADP), IDP, and GDP, with reaction rates relative to that for ADP of 39, 29, and 4%, respectively [12]. Establishment of a more sensitive assay procedure for creatine using CK than the conventional method would be particularly valuable because the method could be used to improve the detection of serum creatinine and may enable earlier diagnosis of kidney dysfunction [13]. The proposed reaction scheme is shown in Fig. 1. ATP and IDP were chosen as the nucleotide pair. If the CK cycling reaction occurs in the presence of excess ATP and IDP, both ADP and ITP will accumulate as the reaction proceeds. Then, using a method that selectively detects ADP

Abbreviations used: CK, creatine kinase; CP, creatine phosphate; PK, pyruvate kinase; LDH, lactate dehydrogenase; 3α-HSD, 3α-hydroxysteroid dehydrogenase; dADP, deoxyadenosine diphosphate; ADP–GK, ADP-dependent glucokinase; G6PD, glucose-6-phosphate dehydrogenase; GK, glycerokinase; GPO, L-α-glycerophosphate oxidase; CNH, creatinine amidohydrolase; Pipes, piperazine-1,4-bis(2-ethanesulfonic acid; Popso, piperazine-1,4-bis(2-hydroxy-3-propanesulfonic acid) dehydrate; Bicine, *N,N*-bis(2-hydroxyethyl)glycine; TODB, *N,N*-bis(4-sulfobutyl)-3-methylaniline, disodium salt; EGTA, *O,O'*-bis(2-aminoethyl)ethylene glycol-*N,N,N',N'*-tetraacetic acid.

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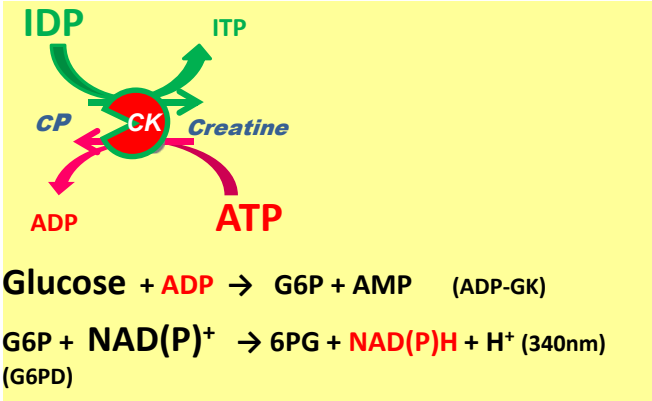

Fig.1. Reaction scheme for the CK cycling reaction in the presence of ADP–GK and G6PD. ADP, generated by the CK cycling, was measured from the reduced NADP at 340 nm.

or ITP, the real-time detection of the reaction can be accomplished. Each reaction mediated by CK in Fig. 1 is as follows:

$$\text{Creatine} + \text{ATP} \rightarrow \text{CP} + \text{ADP} \quad (1)$$

$$\text{CP} + \text{IDP} \rightarrow \text{creatine} + \text{ITP}. \quad (2)$$

The sum of reactions (1) and (2) is

$$\text{ATP} + \text{IDP} \rightarrow \text{ADP} + \text{ITP}.$$

In our experiments, we chose to use ADP-dependent glucokinase (ADP–GK) from *Pyrococcus furiosus* because the reactivity of the enzyme for IDP and GDP is reportedly very low (2–3% that for ADP) [14]. ADP, the product of the reaction, is measured by ADP–GK and glucose-6-phosphate dehydrogenase (G6PD). Here, we report the first use of CK cycling for the quantitative determination of creatine. In addition, a preliminary study of the application of CK cycling to develop a creatinine assay is also described.

Materials and methods

Chemicals

Rabbit muscle CK and NAD(P)$^+$ were obtained from Oriental Yeast (Tokyo, Japan). CK from human muscle, ADP–GK from *P. furiosus*, glycerokinase (GK) from *Elizabethkingia meningoseptica,* and L-α-glycerophosphate oxidase (GPO) from *Streptococcus* sp. are the products of Asahi Kasei Pharma Corporation (Tokyo, Japan). Nucleotides, such as ATP, ADP, dADP, GDP, and IDP, and peroxidase from horseradish were obtained from Sigma. G6PD from *Leuconostoc mesenteroides* and creatinine amidohydrolase (CNH) from microorganism were obtained from Toyobo (Osaka, Japan). Piperazine-1,4-bis(2-ethanesulfonic acid) (Pipes), piperazine-1,4-bis(2-hydroxy-3-propanesulfonic acid) dihydrate (Popso), *N,N*-bis(2-hydroxyethyl)glycine (Bicine), *N,N*-bis(4-sulfobutyl)-3-methylaniline, disodium salt (TODB), and *O,O'*-bis(2-aminoethyl) ethylene glycol-*N,N,N′,N'*-tetraacetic acid (EGTA) were obtained from Dojindo Laboratories (Kumamoto, Japan). 4-Aminoantipyrine was obtained from Wako Pure Chemical (Osaka, Japan). Human serum was purchased from BizCom Japan (Tokyo, Japan).

Reagents and assay procedure

Nucleotide specificity of the reverse reaction of CK was measured by using a solution containing 50 mM Pipes (pH 6.5), 5 mM CP, 1 mM nucleoside diphosphate, 10 mM glycerol, 5 mM $MgCl_2$, 5 U/ml GK, and 20 U/ml GPO, 4-aminoantipyrine, TODB, and peroxidase. The method is based on the broad specificity of *E. meningoseptica* GK for nucleotide triphosphates, which we confirmed experimentally (i.e., the reaction rates for dATP, GTP, and ITP were 66, 28, and 59% relative to that for ATP). The GK amount (5 U/ml) was determined from the study with GTP. Products of the corresponding triphosphate by the reverse reaction in the assay were detected via glycerol-3-phosphate generated by the action of the GK in the presence of glycerol. Glycerol-3-phosphate and oxygen were then converted into dihydroxyacetone phosphate and hydrogen peroxide by GPO. The rate of hydrogen peroxide formation was readily measured as a color change at 546 nm in the presence of peroxidase and a chromogen. The increasing rate of color development after the addition of 0.01 U/ml CK was then measured.

The CK cycling reaction was initiated by adding IDP to 1 ml of solution containing 50 mM Pipes–NaOH (pH 7.0), 10 mM $MgCl_2$, 10 mM glucose, 1 U/ml ADP–GK, 1 U/ml G6PD, 1 mM $NADP^+$, 8 mM *N*-acetyl-L-cysteine, ATP, and CK from rabbit muscle at 37 °C in the presence of the indicated amount of creatine unless otherwise mentioned. Changes in the absorbance of reduced NAD(P) at 340 nm were measured using a Shimadzu UV-2550 spectrophotometer (Shimadzu, Kyoto, Japan).

The evaluation of linearity and limit of detection of creatine, assay of human serum creatine, and creatinine recovery study of human serum were conducted as follows. Briefly, a mixture of 0.006 ml of sample and 0.24 ml of reagent 1 containing 24 mM Bicine–NaOH (pH 8.5), 12 mM $MgCl_2$, 12 mM glucose, 3 mM IDP, 1.5 mM $NADP^+$, 2 U/ml ADP–GK, 2 U/ml G6PD, and 2 U/ml CNH was mixed and incubated for 5 min at 37 °C in the reaction cuvette. A 0.06-ml aliquot of reaction mix 2 containing 120 mM Pipes–NaOH (pH 6.5), 2 mM ATP, 5 mM EGTA, and 1000 U/ml CK from rabbit muscle was then dispensed into the cuvette and incubated for an additional 5 min. The reaction was monitored by measuring the absorbance at 340 nm for 10 min (34 points) using a Hitachi 7170S clinical analyzer (Hitachi, Tokyo, Japan).

Results

Compared with the reaction using ADP at pH 6.5 (100%), the relative velocities of the reaction using dADP, GDP, and IDP were 86, 7, and 13%, respectively. These relative ratios differ slightly from those reported by O'Sullivan and Cohn [12], possibly due to variation in the reaction conditions (e.g., detection method).

As a precondition for the enzymatic cycling reaction, the initial concentration of analyte (CP or creatine in Fig. 1) is proportional to the rate of the individual products generated from the excess substrate (ATP and IDP) [7,8]. When the cycling rate constant is designated as k_c (1/min),

$$d\,[\text{ITP}]/dt = d[\text{ADP}]/dt = k_c[\text{CP} + \text{creatine}]_0, \quad (3)$$

where $[\text{CP} + \text{creatine}]_0$ is the sum of the initial concentration of CP and creatine (see Fig. 1). When the first-order rate constant for each direction of the reaction is represented as k_1 and k_2, k_c is expressed as

$$k_c = k_1k_2/(k_1 + k_2) \quad (4)$$

(see Refs. [7,8]). The integrated equation is

$$[\text{ITP}] = [\text{ADP}] = k_c\,[\text{CP} + \text{creatine}]_0\,t. \quad (5)$$

When the concentrations of ATP and IDP are in excess compared with the analyte, the initial rate of the reverse reaction involving