Journal of Chromatography, 612 (1993) 156–160
Biomedical Applications
Elsevier Science Publishers B.V., Amsterdam

CHROMBIO. 6630

Short Communication

Biocytin synthetase activity in human milk as assessed by high-performance liquid chromatography

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(First received June 12th, 1992; revised manuscript received October 8th, 1992)

ABSTRACT

A reversed-phase liquid chromatographic assay for biocytin synthetase activity has been developed. By this method, biocytin synthetase, isolated to homogeneity from human milk, was found to synthetize biocytin from biotin and L-lysine in the presence of ATP and magnesium ion(s). Both ATP and magnesium ion(s) were required for the synthesis of biocytin. Equal molar amounts of ADP and ATP were produced and consumed, respectively, in the course of the production of the same molar amount of biocytin; however, production of AMP was not observed. Biocytin synthetase Michaelis constants were 2.5, 1.8, and 0.11 m*M* for biotin, L-lysine, and ATP, respectively. Biocytin synthetase from milk was shown to synthesize biocytin in a stoichiometric amount.

INTRODUCTION

Biotinidase or biocytin hydrolase (EC 3.5.1.12) is widely present in various tissues and body fluids, and hydrolyses mainly biocytin and very short biotinyl peptides [1]. Using partially purified biotinidases from bacteria such as *Lactobacillus casei* and from various tissues, Knappe *et al.* [2] and Pispa [3] demonstrated the production of trace amounts of biocytin from biotin and L-lysine. However, the significance of this finding has not been discussed.

Recently, the importance of biocytin accumulation in patients with biotinidase deficiency was underscored by Wolf *et al.* [4]. Biocytin has been considered to be a final catabolic product from four kinds of biotin-containing carboxylase, *i.e.* pyruvate carboxylase, 3-methylcrotonyl-CoA carboxylase, acetyl-CoA carboxylase, and propionyl-CoA carboxylase [1], and of other unknown biotin-containing proteins [5]. Biocytin is then hydrolysed by biotinidase to biotin and L-lysine. Biocytin injected into blood was found to overflow into urine in cases of biotinidase deficiency [6].

In order to clarify further the mechanism of synthesis of biocytin, we measured biocytin by high-performance liquid chromatography (HPLC). We found chromatographically that human milk was able to synthesize biocytin. This paper describes the use of HPLC to demonstrate quantitatively the synthesis of biocytin in milk from biotin and L-lysine, in the presence of ATP.

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0378-4347/93/$06.00

EXPERIMENTAL

Chemicals and reagents

Biotin, biocytin (biotinyl-lysine, Bct), biotinyl-*p*-aminobenzoate (BPAB), *p*-hydroxymercuribenzoate (PHMB) and EDTA were purchased from Sigma (St. Louis, MO, USA). ATP, ADP, AMP and GTP were from Boehringer-Mannheim Yamanouchi (Tokyo, Japan). 9-Anthryldiaminomethane (ADAM) was from Funakoshi Pharmaceutical (Tokyo, Japan). L-Lysine, L-lysine hydrochloride, magnesium chloride and mercuric chloride were from Wako (Osaka, Japan).

Specimens

Human milk was obtained from Tokyo Boshihoken-in Hospital (Tokyo, Japan) and stored at −20°C.

Determination of biocytin (biotinyl-lysine) by reversed-phase HPLC

Biocytin was assayed by reversed-phase HPLC system as follows. A Nucleosil $5C_{18}$ column (250 mm × 4.6 mm I.D., Macherey-Nagel, Düren, Germany) was thermostated at 45°C by a column oven (Model 655A-52, Hitachi, Tokyo, Japan). An HPLC pump with a gradient-making unit (Model 2150 HPLC pump with 2152 LC controller, Pharmacia-LKB, Uppsala, Sweden) was used. Sample injection was carried out with a Model U6K universal liquid chromatography injector (Millipore, Milford, MA, USA) with a 1-ml loop. Linear gradient elution from solvent A (0.1 *M* sodium phosphate buffer, pH 2.1) to solvent B (solvent A–methanol, 20:80, v/v) in 15 min was employed. The flow-rate was 1.5 ml/min, and the column-inlet pressure was *ca.* 80–120 kg/cm^2. L-Lysine was eluted at the flow-through fraction with a retention time of *ca.* 4.0 min. Biotinyl-lysine and biotin were retained on this reversed-phase column and eluted with retention times of 7.1 and 9.2 min, respectively. Biotin and biocytin were detected (Model 655A-21, Hitachi) at 210 nm and 0.08 a.u.f.s. Detection of biocytin and biotin was possible when amounts of 100 and 500 ng, respectively, were injected (signal-to-noise ratio of 3).

Biocytin synthesizing reaction

The reaction mixture (0.1 ml) was composed of 0.01 ml of enzyme solution, 0.01 ml of 10 m*M* ATP, 0.01 ml of 10 m*M* magnesium chloride, 0.01 ml of 50 m*M* L-lysine hydrochloride, 0.01 ml of 50 m*M* biotin (dissolved by neutralization with 1 *M* NaOH) and 0.05 ml of 0.1 *M* Tris–HCl buffer (pH 7.1). After the reaction at 37°C, 0.1 ml of 0.1 *M* HCl was added to stop the reaction. Following centrifugation at 1500 *g* for 5 min, a portion (0.05 ml) of the resultant supernatant was injected into the HPLC system.

Biotinidase activity

Biotinidase activity was assayed using BPAB as a substrate as previously described [7].

Amino acid and biotin analysis

Synthesized biocytin was hydrolysed by 6 *M* HCl at 110°C for 18 h under reduced pressure (by use of a vacuum pump) and the amino acids were assayed by an HPLC amino acid analyser [8]. The biotin content in the hydrolysate was determined by the fluorimetric derivatization method with ADAM reagent (0.1% ADAM in ethyl acetate) as described previously [9]: the excitation wavelength was 365 nm, and the emission wavelength 412 nm.

Inhibitor tests

Inhibitors (metal ions and ATP analogues) were dissolved in distilled water. After incubation of the inhibitor with biotinidase at 23°C for 15 min, the reaction was started by adding the reaction mixture.

Kinetic study

Michaelis constants (K_m) were determined by the method of Lineweaver and Burk [10].

Biotinidase isolation

Biotinidase from milk was purified using BPAB as a substrate. Human milk biotinidase (relative molecular mass 68 000) was purified essentially as previously described by Oizumi *et al.* [8]. Isolated biotinidase was dialysed against 20 m*M* Tris–HCl buffer (pH 7.1) containing 1 m*M* EDTA, 4 m*M* magnesium chloride and 10% glycerol, and dialysed enzymes were stored at −20°C.

Purification of biocytin synthetic activity from milk

Biocytin synthetic activity was purified from 330 ml of milk by the same procedure as for milk biotinidase [8]. Biocytin formation was assayed by the HPLC method as described above.

AMP assay

AMP was assayed by salting-out chromatography using the Nucleosil $5C_{18}$ column. The mobile phase was 0.1 *M* sodium phosphate buffer (pH 2.1) containing 1 *M* sodium sulphate, and the flow-rate was 1.0 ml/min. AMP was eluted at a retention time of 6.4 min and detected by fluorimetric detection (excitation 276 nm, emission 365 nm; Model RF-550 spectrofluorimetric detector, Shimadzu, Kyoto, Japan).

ATP and ADP assay

ATP and ADP were separated by an anion-exchange column of Nucleosil NH_2 (150 mm × 4.6 mm I.D., Macherey-Nagel). The mobile phase was 0.1 *M* sodium phosphate buffer (pH 2.0) containing 0.3 *M* NaCl. The flow-rate was 1.0 ml/min. AMP, ADP and ATP were eluted at 2.32, 3.13 and 8.63 min, respectively. Detection was again fluorimetric. The coefficients of variation (C.V.) for measurements of 30 pmol ATP and ADP (peak height method) were 0.8 and 0.7%, respectively. Detection limits for nucleotides were 3 pmol at a signal-to-noise ratio of 3.

Sodium dodecyl sulphate polyacrylamide electrophoresis (SDS-PAGE)

SDS-PAGE analysis of proteins was performed by the method of Laemmli [11].

Protein determination

Protein determination was performed with a BCA protein assay kit (Pierce, Rockford, IL, USA).

Amino acid composition analysis

Amino acid compositions were analysed for the purified and dialysed enzymes as described previously [8].

Edman degradation analysis

Amino acid sequences were analysed as previously described [8] by using Applied Biosystem Model 471A.

RESULTS AND DISCUSSION

Human milk was tested for the production of biocytin by the HPLC method described in Experimental. An increase in the peak height of biocytin was observed by reversed-phase HPLC analysis when 5 m*M* biotin, lysine and ATP were added to the reaction mixture (0.1 ml). The apparent specific activity was calculated to be 62.3 pmol/min per mg of protein, which was similar to the activity for BPAB hydrolysis by milk (45.1 pmol/min per mg of protein).

Biocytin synthetase was purified from human milk by a similar procedure as for milk biotinidase [8]. Biocytin synthetase was enriched 1252-fold after six steps of purification, whereas biotinidase activity was enriched 3991-fold by the same purification method.

SDS-PAGE analyses showed that fractions exhibiting biocytin synthetic activity at the final step of purification contained only a protein band of molecular mass 68 000 (data not shown). This molecular mass coincided with that of milk biotinidase as reported previously [8]. The amino acid composition of biocytin synthetase was the same as that reported for biotinidase [8]. Furthermore, the amino acid sequence from the N-terminal to the third amino acid of biocytin synthetase was found to be identical with that of biotinidase. These results suggest that biocytin synthetic activity might be associated with the same protein as the biotinidase [12].

The chromatogram for both the reduction of biotin and the production of biocytin by biocytin synthetic enzyme is shown in Fig. 1. At time zero, no product was detected (Fig. 1A). After 15 min reaction at 37°C, the formation of the 7.1-min peak was observed (Fig. 1B). The 7.1-min peak was collected and lyophilized. The 7.1-min peak fraction obtained was then acid-hydrolysed, and the hydrolysate was analysed for lysine by HPLC–amino acid analysis. The biotin content

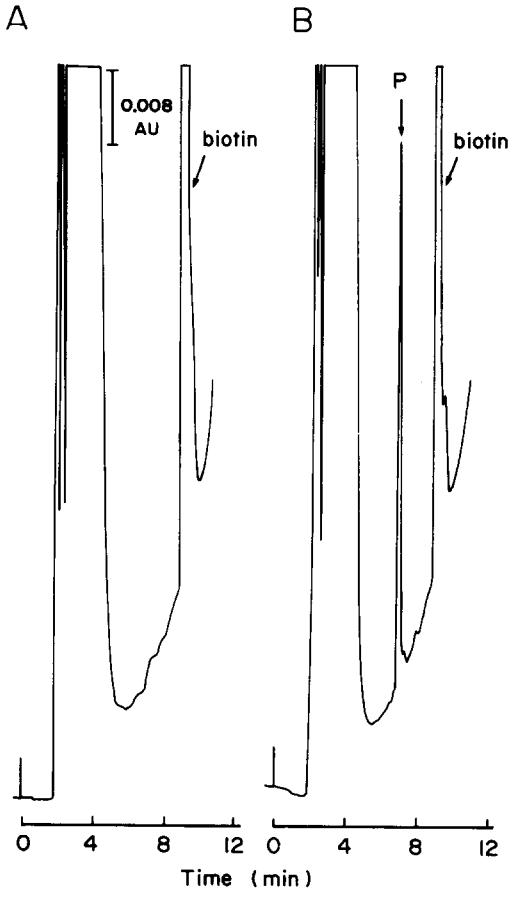

Fig. 1. Typical chromatograms indicating the production of biocytin mediated by biocytin synthetase. (A) Sample at time zero. (B) Biocytin produced by the enzyme after 15 min of incubation. P = product. The supernatant (0.05 ml) of the reaction mixture was injected into the HPLC system. The reaction conditions and analytical conditions were as described in Experimental, except that 5 μg of biocytin synthetase was used.

in the hydrolysate was also determined by the derivatization method with the ADAM reagent [9]. Both biotin and lysine were quantitatively (1:1 molar ratio) detected in the acid hydrolysate. Thus, biocytin synthetase activity was shown to be assayed by HPLC.

The omission of components from complete reaction mixtures was tested, and the need for ATP and magnesium was demonstrated. GTP could not replace ATP at all.

The optimum pH for biocytin synthesis was found to be 6.0–8.0 (data not shown), which was similar to biotinidase activity [8]. The thermal stabilities of biocytin synthetase and biotinidase

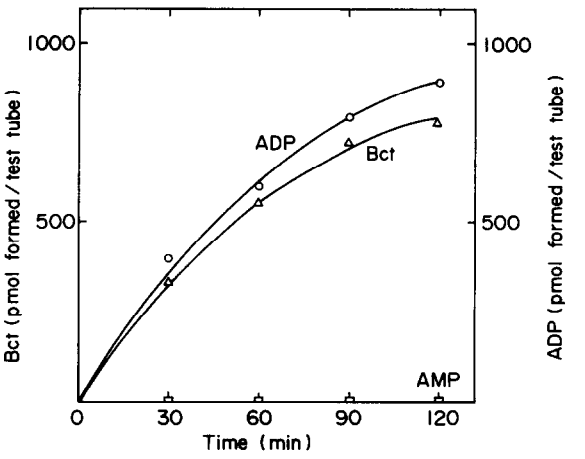

Fig. 2. Time-course study for biocytin (Bct) formation and ADP formation. Each millilitre of reaction mixture contained 20 μg of biocytin synthetase, 50 nmol of biotin, 50 nmol of L-lysine, 10 nmol of ATP, 10 nmol of magnesium chloride, 1 nmol of EDTA, 10 nmol of 2-mercaptoethanol and 100 nmol of Tris–HCl (pH 7.1). At the time indicated, a 0.1-ml portion of the reaction mixture was withdrawn, and 0.1 ml of 0.1 *M* HCl was added to stop the reaction. Other conditions for determination are described in Experimental.

were also similar, *i.e.* 40% residual activity remained after heat treatment at 60°C for 15 min [8].

Because ATP was required for the biocytin synthesis, formation of ADP and AMP was studied (Fig. 2). Biocytin and ADP were formed linearly for 1 h, but no AMP was formed. The stoichiometry of biocytin formation was further studied by changing ATP concentrations. As shown in Table I, stoichiometric formation of

TABLE I

STOICHIOMETRY OF BIOCYTIN FORMATION

Values are nmol formed or reduced per hour per 0.1 ml of reaction mixture. The reaction mixture (0.1 ml) contained 10 μg of biocytin synthetase, 5 nmol of biotin and 5 nmol of L-lysine. Magnesium ions were added in equimolar amounts with respect to ATP. Other conditions were as described in Experimental.

Conditions	Biocytin formed	ADP formed	AMP formed	ATP decrease
ATP, 1.0 m*M*	3.60	4.32	0.0	N.D.
ATP, 0.25 m*M*	3.45	3.97	0.0	4.39
ATP, 0.10 m*M*	3.23	3.43	0.0	3.57

biocytin and ADP was demonstrated at ATP concentrations between 0.25 and 0.10 m*M*. Equimolar amounts of ATP decrease and of ADP increase occurred at 0.10 m*M* ATP.

Kinetic studies for synthetic reaction was performed. The K_m values for biotin, lysine and ATP were 2.5, 1.8 and 0.105 m*M*, respectively. Although K_m for lysine was not described, Shenoy and Wood [13] reported very small K_m values of 0.002 and 0.038 m*M* for biotin and ATP, respectively, in the case of holocarboxylase synthetase.

Mercuric chloride and PHMB completely inhibited the biocytin synthetase activity at 0.001 m*M* concentration. Biotinidase is also inhibited by mercurials at 0.005 m*M*, and belongs to a group of thiol-type hydrolases [8]. Biocytin synthetase might also be classified as a thiol-type enzyme.

Thus, we demonstrated the synthesis of biocytin in human milk with the described HPLC method. Furthermore, biocytin synthesis by biocytin synthetase was shown to require a stoichiometric molar amount of ATP by combination of two HPLC methods: reversed-phase and anion-exchange.

ACKNOWLEDGEMENT

This work was supported by the Ministry of Health and Welfare, Japan.

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