ELSEVIER

Journal of Chromatography B, 707 (1998) 295–300

JOURNAL OF CHROMATOGRAPHY B

Short communication

Ion-exchange column chromatographic method for assaying purine metabolic pathway enzymes

Herbert Ward[a,*], Douglas Baldwin[a], Tingchung Wang[b], Huber Warner[c], Katherine Seymour[d], Cathleen Marquardt[a], Edward McFalls[e], John E. Foker[a]

[a]*Department of Surgery, University of Minnesota and Veterans Affairs Medical Center, Minneapolis, MN 55417, USA*
[b]*University of Rochester, Rochester, NY 14642, USA*
[c]*National Institute on Aging, National Institutes of Health, Bethesda, MD 20892, USA*
[d]*Veterans Affairs Medical Center, Seattle, WA 98108, USA*
[e]*Department of Medicine, University of Minnesota and Veterans Affairs Medical Center, Minneapolis, MN 55417, USA*

Received 14 April 1997; received in revised form 11 November 1997; accepted 13 November 1997

Abstract

High energy phosphate levels fall rapidly during cardiac ischemia and recover slowly (more than one week) during reperfusion. The slow recovery of ATP may reflect a lack of purine metabolic precursors and/or increased activity of purine catabolic enzymes such as 5′-nucleotidase (5′-NT, EC 3.1.3.5) and adenosine deaminase (ADA, EC 3.5.4.4). The activity of enzymes involved in both the catabolism of ATP precursors (5-NT and ADA) and the restoration of ATP from slow synthetic pathways [adenosine kinase (AK, EC 2.7.1.20), adenine phosphoribosyl transferase (APRT, EC 2.4.2.7) and hypoxanthine phosphoribosyl transferase (HPRT, EC 2.4.2.8)] may directly affect the rate of ATP recovery. Strategies to enhance recovery will depend on the relative activity of these enzymes following ischemia. Their activity in different species and their response to ischemia are not well characterized. Hence, rapid assay methods for these enzymes would facilitate detailed time course studies of their activities in postischemic myocardium. We modified a single ion-exchange column chromatographic method using DEAE-Sephadex to determine the products of incubation of 5′-NT, AK, APRT and HPRT with their respective substrates. The uniformity of the final product measurement procedure for all assays permits the activities of the four enzymes to be rapidly determined in a single tissue sample and facilitates the study of a large number of samples. This technique should also be useful for enzymes of the pyrimidine metabolic pathway.

Keywords: Enzymes; Purine; 5′-Nucleotidase; Adenosine kinase; Adenine phosphoribosyl transferase; Hypoxanthine phosphoribosyl transferase

1. Introduction

ATP provides the energy for myocardial contraction and cellular integrity. High energy phosphate levels are depressed by cardiac ischemia and recovery may require greater than one week in intact animals [1]. We and others [1–5] have reported that inhibition of purine nucleotide and nucleoside catabolic enzymes such as 5′-nucleotidase (5′-NT, EC 3.1.3.5) and adenosine deaminase (ADA, EC 3.5.4.4) can decrease the rate of ATP loss during myocardial ischemia. During reperfusion, ATP can

*Corresponding author.

0378-4347/98/$19.00
PII S0378-4347(97)00577-X

be regenerated from adenosine via adenosine kinase (AK, EC 2.7.1.20), from adenine via adenine phosphoribosyl transferase (APRT, EC 2.4.2.7), from hypoxanthine via a salvage pathway requiring hypoxanthine phosphoribosyl transferase (HPRT, EC 2.4.2.8), or de novo [1]. Thus, purine metabolic pathway enzymes play a critical role in recovery from myocardial ischemia, yet their activities in different species and their response to ischemia are not well characterized.

To understand the dynamics of postischemic ATP recovery in myocardium, knowledge of the activities of the aforementioned competing metabolic pathways is needed. This requires the assay, in each tissue sample, of several enzymes with similar substrates and products. Assays for these enzymes have been well described, but are methodologically varied. Those that measure inorganic phosphate are best suited to purified enzymes because it may be difficult to detect small differences in tissue samples [6]. High-performance liquid chromatography (HPLC) for detection of inorganic phosphate is more sensitive and newer techniques are relatively rapid (1 min) [7–10]. Kinetic methods, such as those that measure 5′-NT activity from the oxidation of NADH using exogenous ADA and glutamate dehydrogenase, are accurate but not adaptable to anabolic enzymes [11]. Radioisotopic monitoring of product formation from radiolabeled substrate by paper or thin layer chromatography is sensitive and facilitates quantification of enzyme activity regardless of purity, concentration, or the presence of tissue constituents [12]. Nevertheless, separating the radiolabeled product from the substrate by these techniques can be time-consuming and cumbersome.

In studies of ATP recovery kinetics, it is desirable to determine the activities of several purine metabolic pathway enzymes in each tissue sample. Furthermore, elucidation of the time course of ATP recovery demands the study of a large number of myocardial tissue samples in each experiment. Because different products are generated by each reaction, the determination of each enzyme activity in a given sample requires distinct incubation conditions. We sought to develop a single assay method that would be capable of rapidly determining concentrations of the products generated from each set of enzyme specific incubation conditions. The final assay step in the determination of the activities of four different enzymes would be the same.

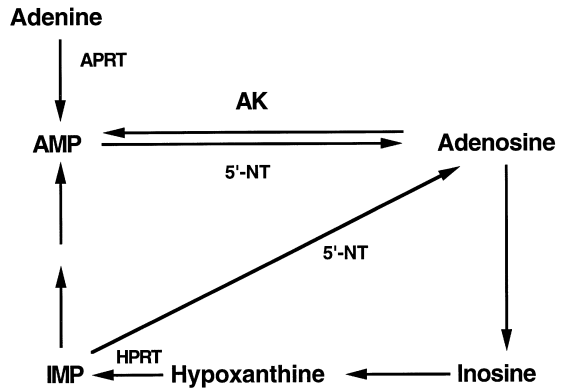

Fig. 1. Purine metabolic pathway enzymes.

To achieve this goal, we modified the method of Warner et al. [13] for determining 5′-NT activity in bacteria to produce assays for the enzymes which catalyze the conversion of purine bases or nucleosides to nucleotides, or vice versa (Fig. 1). Our method uses DEAE-Sephadex in a rapid, simple, single pass ion-exchange column. This method can determine product concentrations resulting from the activities of each of the four enzymes associated with both the catabolism and synthesis of adenine nucleotides. We knew that the presence or absence of the phosphoryl group allowed the separation of products and substrates on Dowex-1 resins. Therefore, the activities of 5′-NT, AK, APRT and HPRT could all be assayed by similar methodology, but purines and purine nucleosides tended to bind nonionically to the resin. Thus, we chose to use DEAE-Sephadex as the ion exchange resin because it is free of aromatic groups. Hence, the separation of the radiolabeled products of each of these enzyme reactions from their substrates can be quickly accomplished with excellent speed and sensitivity in each assay [14–16].

2. Experimental

2.1. Sample preparation

Myocardial biopsies were obtained using a precooled high speed drill, and were frozen within one

second in liquid nitrogen-cooled 2-methylbutane [1]. The biopsies were processed within 24 h of sampling by pulverization in a dry ice cooled mortar followed by homogenization in 50 m*M* Tris–HCl buffer (pH 7.4) containing 3 m*M* $MgCl_2$ and 150 m*M* KCl, using a Micrometrics microhomogenizer. The homogenate was centrifuged at 50 000 *g* for 20 min at 4°C, and the supernatant was decanted and used in AK, APRT and HPRT assays. The pellet was resuspended in the original volume of the homogenization buffer at pH 9.0 (5–10 mg/ml) and used in 5′-NT assays. Samples could be stored at −70°C for up to three months without appreciable loss of activity, but 5′-NT tended to lose activity with refreezing and rethawing.

2.2. Column chromatography

Glass columns (15 cm×0.3 cm I.D. with an 8 cm×1.5 cm I.D. reservoir) were prepared by the glass technology laboratory at the University of Minnesota. Columns were supported in plastic racks designed to hold 20 columns, therefore, at least 20 tissue samples could be assayed simultaneously. Separations were achieved by loading reaction mixtures onto 3 cm×0.3 cm I.D. columns of DEAE-Sephadex which had been previously equilibrated with 10 m*M* ammonium formate (pH 4.3).

2.3. Assay conditions

2.3.1. 5′-Nucleotidase (EC 3.1.3.5)

Approximately 100 μg of the 50 000 *g* pellet fraction was incubated for 10 min at 37°C in a reaction mixture containing 25 m*M* HEPES buffer (pH 7.4) containing 5 m*M* $MgCl_2$, 26 m*M* DTT and 625 μ*M* [^{3}H]AMP (30 nCi) in a total volume of 80 μl. The reaction was stopped by the addition of 0.6 ml of ice-cold 20 m*M* ammonium formate (pH 4.3), and the mixture immediately loaded onto the column. Reaction blanks had cold ammonium formate added prior to the substrate and were rapidly loaded onto the column.

The reaction product, [^{3}H]adenosine, was readily eluted from the column in 20 m*M* ammonium formate, and the column was washed with 1 ml of distilled H_2O. The eluate was collected in counting vials and mixed with 10 ml of scintillation counting fluid (0.17 g 1,4-*bis*[2-(4-methyl-5-phenyloxazolyl)]-benzene, 5.67 g 2,5-dithenyloxazole, 667 ml toluene, 333 ml Triton X-100) and counted at 30% counting efficiency in a Beckman LS 230 counter.

2.3.2. Adenosine kinase (EC 2.7.1.20)

Approximately 100 μg of 50 000 *g* supernatant was incubated for 15 min at 37°C with a reaction mixture containing 20 m*M* Tris maleate buffer (pH 5.75) containing 0.5 m*M* $MgCl_2$, 0.15 m*M* *erythro*-9-(2-hydroxy-3-nonyl) adenine (EHNA), 0.5 m*M* ATP, 1 m*M* DTT and 50 μ*M* [^{3}H]adenosine (60 nCi) in a total volume of 80 μl. The reaction was stopped with 0.6 ml of ice-cold 20 m*M* ammonium formate (pH 4.3), and the mixture loaded onto the column. Reaction blanks were included as above.

The reaction product, [^{3}H]AMP, was initially bound to the column and the remaining substrate, [^{3}H]adenosine, was eluted with 1 ml H_2O and discarded. The reaction product was then eluted by washing the column twice with 0.6 ml of 400 m*M* ammonium formate (pH 4.3). The eluate was collected in the scintillation vial and counted.

2.3.3. Adenine phosphoribosyl transferase (EC 2.4.2.7)

Approximately 200 μg of 50 000 *g* supernatant fraction was incubated for 4 min at 37°C with a reaction mixture containing 44 m*M* Tris–HCl buffer (pH 7.7) containing 4.4 m*M* $MgCl_2$, 400 μ*M* αβ-methylene adenosine 5′-diphosphate (AMP-CP), 1 m*M* phosphoribosyl pyrophosphate (PRPP) and 100 μ*M* [^{3}H]adenine (30 nCi) in a total volume of 80 μl. The reaction was stopped with 0.6 ml of ice-cold 20 m*M* ammonium formate (pH 4.3), and the mixture loaded onto the column. Reaction blanks were included as above.

The reaction mixture was allowed to drain and the column was washed with an additional 0.6 ml of 20 m*M* ammonium formate and 1 ml of H_2O in order to elute substrate. The reaction product, [^{3}H]AMP, was eluted with 0.6 ml of 400 m*M* ammonium formate followed by 1 ml of H_2O. The eluate was collected and counted.

2.3.4. Hypoxanthine phosphoribosyl transferase (EC 2.4.2.8)

The assay for HPRT is identical to that for APRT except that the incubation time is 10 min and the radiolabel added was 600 m*M* [^{3}H]hypoxanthine (50 nCi). The reaction product, [^{3}H]IMP was eluted with 0.6 ml of 400 m*M* ammonium formate followed by 1 ml H_2O, collected and counted.

2.3.5. Protein content

Protein was determined by the method of Lowry et al. [17] using bovine serum albumin as the standard. Samples containing particulate material were first incubated in 1 *M* NaOH for several hours at room temperature.

2.4. Reagents

[^{3}H]AMP (15 Ci/mmol), [^{3}H]adenine (17 Ci/mmol), [^{3}H]adenosine (30 Ci/mmol) and [^{3}H]hypoxanthine (10 Ci/mmol) were purchased from New England Nuclear (Billerica, MA, USA). *erythro*-9-(2-Hydroxy-3-nonyladenine was a gift from Dr. Howard Schaeffer of Burroughs-Wellcome. The αβ-methylene adenosine 5′-diphosphate was purchased from Sigma (St. Louis, MO, USA). Phosphoribosyl pyrophosphate was purchased from Boehringer (Indianapolis, IN, USA). All other chemicals were purchased from Sigma.

3. Results

The specific activities of the enzymes in dog, human and rat hearts from our study are depicted in Table 1, along with the available values reported in the literature. Specific activity is expressed as the number of micromoles of product formed per minute per mg of protein. Activities were obtained from the initial velocities of the enzymatic reactions at saturating substrate concentrations. Apparent K_m values were estimated by least squares approximations of experimental values [18]. All assays proved to be linear with time (except as noted in Section 4) and enzyme concentration under the conditions of the experiments shown.

3.1. 5′-Nucleotidase

This enzyme was most active at a pH of 9.0 but assays were carried out at pH 7.4 to mimic the in vivo situation. Analysis of both fractions indicated that 70% of the activity was in the 50 000 *g* pellet and 30% in the 50 000 *g* supernatant [19,20]. The characteristics of both activities were similar, and the K_m for 5′-AMP in both fractions was 120 μ*M*. Both particulate and soluble activity were inhibited 85% by 400 μ*M* AMP-CP (a specific inhibitor of 5′-NT) [19,20] at pH 7.0, but only inhibited 50% at pH 9.0. If 3′-AMP or glycol phosphate were added as the substrate, no measurable hydrolysis took place.

Table 1
Kinetic parameters of enzymes catalyzing the conversion of purine bases and nucleosides to nucleotides and vice versa

	Refs.	Activity (nmoles/min/mg protein)			
		5′-NT	AK	APRT	HPRT
Dog heart	Present study	5.1±0.6 (10)	0.035±0.002 (6)	0.54±0.06 (5)	0.96±0.12 (5)
	Nakatsu et al. [14]	14.2±0.4	–	–	–
Human heart	Present study	11.2	0.020 (0.015–0.025)	0.39 (0.36–0.42)	1.54 (1.43–1.65)
	Adams et al. [26]	–	–	0.63±0.16	0.55 (0.27–0.83)
Rat heart	Present study	72.3 (69.3–77.8)	0.063 (0.047–0.079)	7.23 (6.94–7.54)	5.13 (5.12–5.15)
	Maguire et al. [16]	–	0.032[a]	41.0[a]	87.0[a]

Values are mean±S.E. in groups with $n \geq 5$ animals sampled or mean with range in brackets in groups with $n < 5$. Number in parenthesis = n for each group. Abbreviations used: 5′-NT, 5′-nucleotidase; AK, adenosine kinase; APRT, adenine phosphoribosyl transferase; HPRT, hypoxanthine phosphoribosyl transferase.

[a] Values were estimated by assuming 10–15 mg protein/g wet weight.

3.2. Adenosine kinase

The optimal pH for this enzyme was 5.7 and the K_m for adenosine was 14 μ*M*. The cytosolic fraction contained greater than 90% of the enzyme activity. In this reaction, 0.15 m*M* EHNA, which inhibits dog myocardium adenosine deaminase (ADA) activity completely, was included to preserve the reaction substrate and prevent its degradation to inosine [2,21]. Inhibitors of Na, K-ATPase (ouabain at 125 μ*M*) and Ca, Mg-ATPase (vanadate at 12 μ*M*) did not alter the apparent AK activity.

3.3. Adenine phosphoribosyl transferase

This enzyme was active over a pH range from 6 to 9. The K_m of APRT for adenine and PRPP was 6 and 65 μ*M*, respectively. AMP-CP was added to the assay in a concentration previously shown to inhibit 85% of 5′-NT activity in order to prevent degradation of the AMP product to adenosine [19]. In the absence of AMP-CP, the measured APRT activity was approximately 1/2 of the activity with AMP-CP, presumably as a consequence of 5′-NT activity.

3.4. Hypoxanthine phosphoribosyl transferase

This enzyme also was active in a pH range from 6 to 9. The K_m of HPRT for hypoxanthine was 120 μ*M*. Again, AMP-CP was added, this time to prevent degradation of IMP to inosine. Both HPRT and APRT had an absolute requirement for PRPP.

4. Discussion

In this report the activities of four purine metabolic enzymes from canine, human and rat myocardium were measured. Currently a variety of assay methods, including inorganic phosphate determination [6,14], spectrophotometry [15] and the use of radiolabeled substrates [12,19,22] with the products separated by thin-layer, paper, or column chromatography [7,19,23–25] are available for these enzymes. All of these methods were designed for measuring the activity of one specific enzyme; to investigate other enzymes in the pathway requires unrelated techniques. The methods described by Warner et al. [13], in which ion-exchange column chromatography is used to assay bacterial 5′-NT activity, is simple, rapid, sensitive and adaptable to assaying other enzymes of this pathway and to large numbers of samples.

In our experience, one person can satisfactorily carry out a mixture of 40–80 5′-NT, AK, APRT and HPRT assays in a six hour period. The number of assays need only be limited by the number of columns available. If greater speed is required, Sephadex can be directly added to tubes containing the reaction mixtures, thoroughly mixed and then separated by low-speed centrifugation. This method of separation has been successful and can increase the number of assays being carried out at one time.

The activities of 5′-NT, APRT and HPRT are easily measurable at nmol/min/mg protein levels. For AK, activities at low as 20 pmol/min/mg protein have been assayed with excellent reproducibility. This procedure appears, therefore, to be highly sensitive.

Our results gave enzyme activities comparable with reported values for dog heart 5′-NT, rat heart AK, human heart APRT and HPRT (Table 1) [14,16,26]. The one difference from previously reported values is our result for rat myocardium APRT and HPRT. The APRT and HPRT activity reported by Maguire et al. [16] in rat myocardium was 6–17-fold higher than our results. In their assay, however, no 5′-NT inhibitors were included and, therefore, relatively short incubation times were used (30 s) to minimize degradation of AMP. Other studies with rat liver have shown that APRT activity has an ‘‘initial burst’’ during the first 10 s in which a fast reaction takes place [27]. This initial rapid reaction, the nature of which is unclear, does not reflect the steady-state activity and may account for the apparent higher APRT and HPRT activities reported by Maguire et al. [16]. In the presence of the 5′-NT inhibitor, AMP-CP, more accurate steady-state values for APRT and HPRT activity can be determined.

The requirement of AK, APRT and HPRT for inhibitors of the catabolic enzymes degrading their substrates or products (ADA or 5′-NT, respectively), and the activity of 5′-NT on 5′-AMP further supports the specificity of the reactions mentioned [20]. In the absence of these inhibitors the measured activities were greatly reduced unless, as previously

discussed for APRT and HPRT, a very short incubation period is used. Moreover, the APRT and HPRT assays have a total dependency on exogenously added PRPP. These requirements further confirm that we are indeed measuring the reactions catalyzed by 5′-NT, AK, APRT and HPRT with the method proposed here.

While we have only demonstrated the usefulness of this technique for assaying enzymes in the purine series, it is reasonable to predict that a similar procedure can be used for the pyrimidine pathways.

Acknowledgements

This work was supported in part by Grants HL 26640 and HL 52157 from the National Institutes of Health and by a Grant-in-Aid from the American Heart Association, Minnesota Affiliate.

References

[1] H.B. Ward, T. Wang, S. Einzig, R.W. Bianco, J.E. Foker, J. Surg. Res. 34 (1983) 292.
[2] J.E. Foker, S. Einzig, T. Wang, J. Thorac. Cardiovasc. Surg. 80 (1980) 506.
[3] H.B. Ward, J.M. Kriett, S. Einzig, R.W. Bianco, R.W. Anderson, J.E. Foker, Surg. Forum 34 (1983) 264.
[4] J.P. Dasmana, S.B. Digerness, J.M. Geckle, J.D. Glickson, E.H. Blackstone, Circulation 64(4) (1981) 275.
[5] W.F. Lubbe, K. Mercer, T. Nguyen, Circulation 64(4) (1981) 96.
[6] B. Glastris, S.E. Pfeiffer, Methods Enzymol. 32 (1974) 124.
[7] A. Amici, M. Emanuelli, N. Raffaelli, S. Ruggieri, G. Magni, Anal. Biochem. 216 (1994) 171.
[8] T. Sakai, S. Yanagihara, K. Ushio, J. Chromatogr. 239 (1982) 717.
[9] E.G.E. Jahngen, E.F. Rossomando, Anal. Biochem. 137 (1984) 493.
[10] L.Z. Ali, D.L. Sloan, J. Biol. Chem. 257 (1982) 1149.
[11] C.L. Arkesteijn, J. Clin. Chem. Clin. Biochem. 14 (1976) 155.
[12] D.R. Janero, C. Yarwood, J. K Thakkar, J. Chromatogr. 573 (1992) 207.
[13] H.R. Warner, R.F. Drong, S.M. Berget, J. Virol. 15 (1975) 273.
[14] K. Nakatsu, G.I. Drummond, Am. J. Physiol. 223 (1972) 1119.
[15] D.M. Goldberg, G. Ellis, J. Clin. Pathol. 25 (1972) 907.
[16] M.H. Maguire, M.C. Lukas, J.F. Rettie, Biochim. Biophys. Acta 262 (1972) 108.
[17] O.H. Lowry, N.J. Rosebrough, A.L. Farr, R.J. Randall, J. Biol. Chem. 193 (1951) 265.
[18] I.H. Segel, Enzyme Kinetics: Behavior and Analysis of Rapid Equilibrium and Steady-State Enzyme Systems, John Wiley and Sons, New York, 1975.
[19] W. Schutz, J. Schrader, E. Gerlach, Am. J. Physiol. 240 (1981) H963.
[20] G.P. Frick, J.M. Lowenstein, J. Biol. Chem. 251 (1976) 6372.
[21] T.W. North, S.S. Cohen, Proc. Natl. Acad. Sci. USA 75 (1978) 4684.
[22] J.N. Stolk, R.A. De Abreu, A.M. Boerbooms, D.G.M. de Koning, R. de Graaf, P.J.S.M. Kerstens, L.B.A. van de Putte, J. Chromatogr. B 666 (1995) 33.
[23] W.J. Arnold, W.N. Kelley, in: P.A. Hoffee, M.E. James (Eds.), Methods in Enzymology, Academic Press, New York, 1978, p. 568.
[24] N. Nakamura, Biochim. Biophys. Acta 426 (1976) 339.
[25] A.D. Olsen, G. Milman, in: P.A. Hoffee, M.E. James (Eds.), Methods in Enzymology, Academic Press, New York, 1978, p. 543.
[26] A. Adams, R.A. Harkness, Biochem. J. 160 (1976) 565.
[27] D.P. Groth, L.G. Young, J.G. Kenimer, in: P.A. Hoffee, M.E. James (Eds.), Methods in Enzymology, Academic Press, New York, 1978, p. 574.