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Fluorometric Microassays of Adenylate Kinase, an Enzyme Important in Energy Metabolism

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ABSTRACT

The adenylate kinase system offers a mechanism for the rapid provision of energy by catalysing the production of ATP from ADP. Fluorometric micromethods were developed for determination of the activity of this enzyme using either formation of ADP or ATP, in each case measured by coupling to suitable dehydrogenase reactions. Both procedures yielded results in good agreement, but when ADP formation was measured an interfering phosphatase splitting of ATP had to be corrected for. Therefore, ADP was preferred as the substrate and its conversion to ATP was determined in a coupled hexokinase–glucose-6-phosphate dehydrogenase reaction yielding stoichiometric amounts of NADPH which were measured by the native fluorescence of this form of the nucleotide. The sensitivity and reproducibility of our micro-method permitted assay of small samples (50–500 ng) such as a layer of cerebellar cortical nerve cells and of insulin producing cells from the islets of Langerhans. Although not reaching the high values in muscle, these cells showed significantly higher activities than parenchymatous cells from the liver and the exocrine pancreas. The sensitivity attained is more than required for assay of clinical fine needle biopsies and is quite satisfactory for detection and estimation of adenylate kinase contaminants in enzyme preparations.

INTRODUCTION

Replacement of ATP can occur by phosphorylation of ADP in the respiratory chain, during glycolysis or by conversions from creatine phosphates. Adenylate kinase (ATP: AMP phosphotransferase, EC 2.7.4.3) can also catalyze fast energy transfer from ADP in the reaction 2 ADP$\rightleftarrows$ATP+AMP. Skeletal and also heart muscle possess very high activities of this enzyme (8, 16) and therefore may be capable of meeting a sudden demand for ATP (18). The immediate energy demands are not, however, limited to muscle movements but also concern other processes such as changes in membrane potentials (4) and the release of transmittor substances (5) and hormones (10). It is worthy of mention that hormonal discharge from certain endocrine cells is surprisingly high as for example the release of insulin from the B-cells in the islets of Langerhans (13).

Nervous or endocrine tissues engaged in the transmission of signals is characterized by a complex structure with only a limited number of the cells involved in the activity thus making it difficult to obtain satisfactorily large samples for analysis. Micromethods are therefore required for analyses of small cell samples such as those which can be prepared from the cerebellar cortex or from endocrine glands such as the islets of Langerhans. Access to material is also severely restricted in clinical examination, using fine needle biopsies for micro analyses. Furthermore sensitive methods are of value in checking enzyme preparations in which adenylate kinase is often a more or less easily detectable contaminant. This report will describe microchemical analyses of adenylate kinase by means of fluorescence techniques.

MATERIAL AND METHODS

Animals. Since the study included analyses of the islets of Langerhans, hyperglycemic obese mice (gene symbol *ob/ob*) were chosen as experimental animals. These are frequently used in islet research because of their comparatively big and hyperactive islets (12, 14).

Tissue samples. In principle the techniques described by Lowry and his collaborators were used for the preparation of the samples (17). The animals were anesthetized with ether and decapitated.

Small pieces of pancreas, liver, kidney, cerebellum and heart muscle were quickly dissected out, placed on cryostat chucks, using brain paste as adhesive, and immediately frozen in isopentane chilled to −160°C with liquid nitrogen. They were subsequently kept at −85°C for 0–5 days until sectioned at 20 μm in a cryostat (−25°C). The sections were then lyophilized at −30°C, and stored at −25°C in vacuum (1.3 Pa) or in argon gas containing less than 1 ppm oxygen.

Cell samples were dissected freehand from freeze-dried sections under a stereomicroscope in a thermostated room

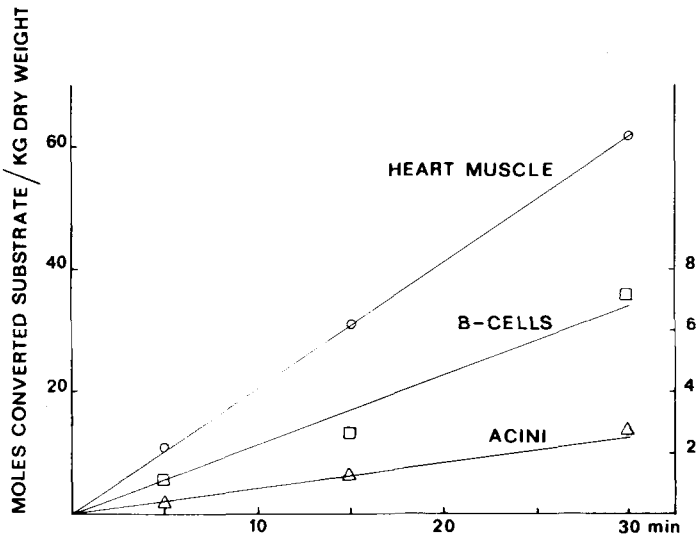

Fig. 1. Relationships between conversion rate and time. The rates given at the ordinate show the ATP formation measured with coupled Hexokinase–G-6-PDH reactions. The scale to the left refers to heart muscle and that to the right to pancreatic exocrine acini and to B-cells from the islets of Langerhans. The abscissa gives the reaction time in min. Each symbol represents the mean of four to five determinations.

at 24°C and 40% relative humidity. Samples in the weight range 50–500 ng were isolated from the central parts of the islets of Langerhans (B-cells), exocrine pancreatic acini, central parts of liver lobules, cortex of kidney (tubules), granular layer of cerebellum and from heart muscles. The samples were weighed on quartz fiber balances, either with a sensitivity of 7 ng/scale division (muscle) or with a sensitivity of 17 ng/scale division (other cell samples). In general, the result for each individual animal was the mean of from three to five samples from each type of preparation.

Chemicals. Triethanoleamine hydrochloride (TRA), NAD, NADH, NADP, NADPH, glucose-6-phosphate (G-6-P), ATP, ADP, AMP and phosphoenolpyruvate (PEP) together with solutions of the enzymes hexokinase (EC 2.7.1.1), glucose-6-phosphate dehydrogenase (G-6-PDH, EC 1.1.1.49), pyruvate kinase (PK, EC 2.7.1.40) and lactate dehydrogenase (LDH, EC 1.1.1.27) were obtained from Boehringer und Soehne, Mannheim. D-glucose was from Mallinckrodt and bovine plasma albumin (BPA) from Miles laboratories. Ethylenediamine tetraacetate (EDTA) and salts which were of analytical grade were obtained from Merck.

ANALYTICAL PERFORMANCE AND EVALUATION OF THE PROCEDURES

Assay of ADP formation. The activity of adenylate kinase is usually measured by ADP production coupled to the PK–LDH reactions (2, 3, 15), but it may also be assayed in the other direction (1, 9, 19). Since the equilibrium constant at physiological pH is claimed to be near unity (6), both assay methods should give approximately the same result.

For measurements of the ADP formation the incubation medium consisted of 9.4 μl of 0.5 M TRA buffer pH 7.6, containing 3 mM AMP, 2.4 mM ATP, 1.4 mM PEP, 1.4 mM NADH, 157 mM KCl, 7 mM $MgCl_2$, 0.05% BPA and 0.3 U/ml of pyruvate kinase and 1.8 U/ml of lactate dehydrogenase. After incubation 15–30 min at 38°C the reaction was stopped by cooling in ice and acidification. Fluorescence from NAD^+ was developed with strong alkali and measured after dilution (17). Appropriate blanks and standards were carried through the entire analytical procedure. The results were expressed as moles substrate consumed per kg sample dry weight per hour at 38°C (MKH units). The reaction rate was linear both with time and with the sample weight in the range 100–500 ng. Interference by phosphatases capable of splitting ATP to ADP was a problem in some analyses which had to be corrected for, by the introduction of tissue blanks processed without AMP in the incubation medium.

Assay of ATP formation. In other experiments the adenylate kinase reaction was run in the other direction thus resulting in ATP formation which was measured as originally suggested by Oliver (19). Coupling to the hexokinase-G-6-PDH reactions yielded NADPH as a measurable product in an assay adopted to the microscale as follows. The samples were incubated 15–30 min at 38°C in 9.4 μl medium consisting of 0.05 M TRA buffer pH 7.6, 1.6 mM NADPH, 2 mM glucose, 3 mM ADP, 1 mM EDTA, 5 mM $MgCl_2$, 0.05% BPA and 0.7 U/ml of hexokinase and 0.3 U/ml of glucose-6-phosphate dehydrogenase. After incubation, the reaction was

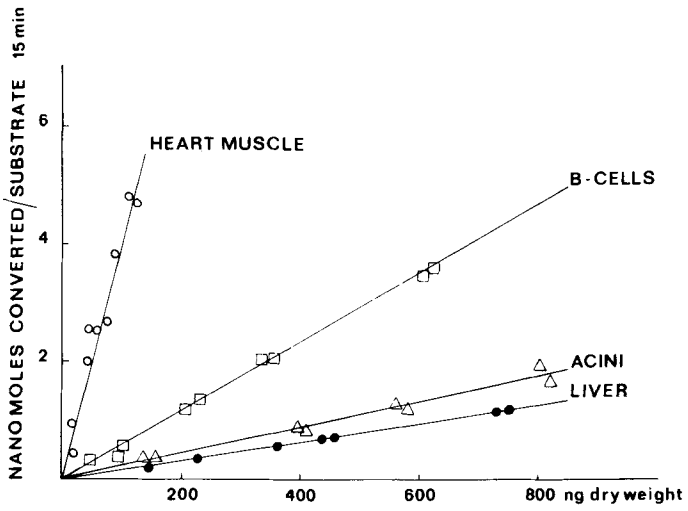

Fig. 2. Relationships between conversion rates and sample weights. The rates on the ordinate show the ATP formation measured with coupled hexokinase–G-6-PDH reactions. Each symbol gives the mean of five determinations.

stopped by cooling in ice and immediately diluted 35 fold with 0.01 mM NaOH containing 5 mM EDTA. The native fluorescence of NADPH was then determined with a Farrand ratio fluorometer or a Turner model 111 fluorometer. Blanks and standards of glucose-6-phosphate and NADPH were treated the same way. Estimates of adenylate kinase activity were made in the pH range 6.7–9.2. The results showed a broad maximum between pH 7 and pH 8.5 for all the cell samples examined.

The reaction was linear with time up to at least 30 min in tests made with samples of appropriate weights, see Fig. 1. The samples were thus incubated either for 15 or 30 min depending on their weights, but only for 15 min in case of heart muscle preparations because of their high adenylate kinase activity. Since ADP is a product of the hexokinase reaction operating in the system, the ADP concentration was well maintained. This was checked to be sufficient for a maximal rate and should exceed the K_M value for ADP manyfold. It has been reported to be in the range of 0.2–0.3 mM in preparations from rat liver (20). The relationship between the sample weight and the consumption of substrate was evaluated as shown in Fig. 2. There was a distinct linear relation in the range 50–800 ng for the different cell populations.

COMPARISONS AND CONCLUDING REMARKS

The results of the two fluorometric methods were compared, using means of 4–5 preparations from various organs of a single animal. The ratios between the MKH values obtained in the compensated PK-LDH assay and in the analyses with the hexokinase–G-6-PDH method were, for the islets 1.09, for the kidney cortical tubules 1.06, for the granular layer of the cerebellum 0.91, and for heart muscle 0.87. The hexokinase–G-6-PDH method is more convenient to handle and is thus preferred. It deserves also be pointed out that the hexokinase reaction prevents accumulation of ATP which would become a substrate for phosphatases possessing higher K_m values than hexokinase (11, 21). The total error of the preferred, enzyme assay including both the method errors and the biological variation was calculated on quintuplicates as the coefficient of variation and found to be $\pm 16\%$.

Assay of minute defined cell samples represents a more advanced application of the methods than detecting adenylate kinase as contaminant in various enzyme preparations. The experiment demonstrated that small groups of cells, engaged in discharge of hormones or signals, can be analyzed. The conversion rates given in Fig. 2 would correspond to about 20 MKH for the B-cells. The adenylate kinase activity of the cerebellar granular layer is about 50% higher, amounting to 30.1 ± 2.6 MKH as determined in 5 animals. This was much below the very high activity of heart muscle but significantly higher than the values in parenchymatous cells from the liver or the exocrine acini of the pancreas. These findings have been confirmed and corroborated (7).

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