A new system for ATP-metric immunoanalysis

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Treatment of amino-group-containing antigens with adenosine-5'-trimetaphosphate results in their chemical modification by -pppA residues. An immunoanalytical system is proposed based upon competition of these ATP-labelled antigens with those of the sample for immobilized antibodies. Mild acidic treatment of complexes of ATP-labelled antigens with immobilized antibodies results in quantitative liberation of intact ATP. The latter may be determined by the ultrosensitive bioluminescent techniques based upon emission of light with firefly luciferase. The validity of the system has been studied with two clinically important antigens, thyroxine and myoglobin.

Myoglobin *Thyroxine* *Immunoanalysis* *ATP* *Luciferase* *Bioluminescence*

1. INTRODUCTION

Much effort is put into elaboration of novel immunoanalytical systems alternative to radioimmunoanalysis. Enzymes, fluorescent and chemiluminescent compounds and free radicals are used as alternative labels [1]. The high sensitivity and the simplicity of the well-known luciferin–luciferase test [2] make ATP an attractive alternative label for immunoanalysis. ATP-metric immunoanalytical procedures have been proposed in [3,4]. According to these procedures, ATP-labelled antigens themselves are taken into the luciferin–luciferase reaction. However, the presence of antigens as substituents at position 6 of the adenosine residue of ATP leads to a considerable and unpredictable decrease of the substrate activity of these derivatives compared with ATP, and consequently to a decrease in the sensitivity of the assay.

Here, we describe a novel ATP-metric immunoanalytical system, the most important feature of which is liberation of intact ATP from the complex of the ATP-labelled antigen with antibodies. Thyroxine and myoglobin were taken as model antigens.

2. MATERIALS AND METHODS

2.1. *Synthesis of adenosine-5'-trimetaphosphate (ATMP)*

This was done as in [5]: 40 mg dry triethylammonium ATP was dissolved in 400 μl dry dimethylsulfoxide; 120 mg dicyclohexylcarbodiimide and 4 mg pyridinium hydrochloride were added, and the mixture kept for 1 h at 25°C. The yield of ATMP as determined in [5] was 100%. Dicyclohexylurea was removed by decantation. The dimethylsulfoxide solution of ATMP thus obtained was stored at −10°C.

2.2. *The synthesis of the ATP γ-amide, the derivative of thyroxine methyl ester (MeT_4-ATP)*

Thyroxine methyl ester (MeT_4) was obtained as in [6]. The structure of the compound was determined by PMR-spectroscopy: 20 mg $MeT_4 \cdot HCl$ was dissolved in 500 μl dry methanol; 100 μl dry triethylamine was added, followed by 100 μl ATMP dimethylsulfoxide solution (section 2.1). The mixture was kept for 30 min at 37°C and poured into 40 ml water. The solution was applied

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to a DEAE-cellulose column, and chromatography was performed in a linear gradient of triethylammonium bicarbonate (TEAB). The peak which was eluted at 0.9 M TEAB was collected and evaporated in vacuo under argon to dryness. The residue was dissolved in water (2 ml), and the solution added dropwise to 50 ml 1% NaI acetone solution saturated with argon. The yield of sodium MeT_4-ATP was 50%.

2.3. *The synthesis of ATP-labelled myoglobin (Mb-ATP)*

This was started with human myoglobin (Mb) purified as in [7]: 0.25 mg Mb was dissolved in 100 μl sodium borate (pH 10); 2 μl ATMP solution (section 2.1) was added. The mixture was kept for 30 min at 37°C and subjected to gel-filtration on Sephadex G-50 in 0.1 M phosphate (pH 7.5). The polymer peak was collected, divided into 100 μl aliquots and kept at −10°C.

2.4. *ATP-metric immunoanalysis for thyroxine*

Rabbit anti-T_4 serum (a gift of Dr T. Loevgren, LKB, Wallac, Turku) was bound with BrCN–Sepharose (Pharmacia, Uppsala): 1 mg immunoadsorbent was suspended in buffer A (0.01 M sodium borate (pH 8.4)–0.15 M NaCl–1% bovine serum albumin); 2.6×10^{-11} mol (40 ng) MeT_4-ATP in buffer A (400 μl) was added to 200 μl immunoadsorbent suspension (5 mg/ml) followed by 200 μl T_4 standard, or unknown human serum, and 2 ml 0.01 M sodium borate (pH 8.4). The mixture was shaken for 12 h at 37°C and centrifuged at 0°C. The supernatant was thoroughly removed. The adsorbent was suspended in 200 μl 0.01 M HCl, kept for 90 min at 37°C, and centrifuged; 100 μl supernatant was mixed with 700 μl 0.1 M Tris–acetate (pH 7.8)–2.8 mM EDTA. The concentration of ATP was determined in the solution obtained using the ATP-monitoring luciferin–luciferase kit no.2 and Luminometer 1250 of LKB Produkter AB (Sweden) according to the manufacturer's instructions; 2.6×10^{-11} mol ATP gave a signal of 200 mV. The signal was directly proportional to the concentration of ATP over a wide range.

2.5. *ATP-metric immunoanalysis for myoglobin*

Rabbit anti-Mb serum was immobilized by UV-irradiation on 9 mm photoreactive paper discs (the preparation of these azido-group-containing discs will be described elsewhere). Antibodies immobilized on a single disc bound about 4 ng Mb. Wet discs were placed into test-tubes, and 0.6×10^{-12} mol (10 ng) Mb-ATP in 100 μl buffer B [0.05 M sodium phosphate (pH 7.5)–0.08 M NaCl–0.01 M EDTA–0.5% bovine serum albumin] followed by 100 μl standard or sample were added. The standards were prepared in the following buffer: 0.05 M sodium phosphate (pH 7.5)–0.08 M NaCl–0.01 M EDTA–4% bovine serum albumin–1% bovine serum immunoglobulin G. Over 6 h gentle shaking at 25°C the reaction mixture was removed by suction, and the disks washed with 500 μl 0.05 M sodium phosphate (pH 7.5)–0.08 M NaCl–0.01 M EDTA–0.2% Twin 20, followed by 1 ml water. The discs were placed into 0.02 M HCl (100 μl) and incubated for 75 min at 40°C in closed test-tubes. After the hydrolysis, 700 μl 0.1 M Tris–acetate (pH 7.8)–2.8 mM EDTA was added, and the mixture incubated during 30 min at 37°C; 200 μl ATP-monitoring mixture [2] was added, and the level of light emission was measured by means of Luminometer 1250.

2.6. *Radioimmunoanalysis*

Radioimmunoanalysis for T_4 and the measurement of the antigenic activity of MeT_4 were performed using the T_4-RIA kit of Farmos Diagnostica (Turku) according to the manufacturer's instructions. Radioimmunoanalysis for Mb has been described [7].

3. RESULTS

It is known that adenosine-5′-trimetaphosphate reacts with amines to give ATP γ-amidates [5]:

Scheme 1

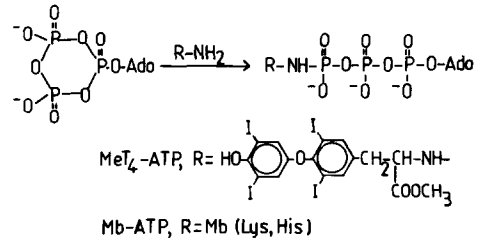

Table 1

Stoichiometry of the labelling of Mb by ATP

Mb-ATP dilution after gel filtration	[Mb] as determined by RIA	[ATP] determined by luminometry after acidic hydrolysis
1:2000	9.8 nM	52 nM
1:4000	5.6 nM	25 nM

We used this reaction to attach the ATP label to antigens. Regeneration of free ATP was performed by mild acidic treatment which cleaves the phosphoamide bond in ATP γ-amidates [5].

The structure of MeT_4-ATP obtained follows from: (i) the method of its synthesis which is known to give ATP γ-amidates [5]; (ii) UV-spectroscopy; (iii) quantitative transformation into MeT_4 and ATP under mild acidic treatment; (iv) immunological properties described below.

ATMP reacts with Lys and His residues in proteins. Modification of Mb under the conditions in section 2 afforded a product which contained 5 -pppA residues/molecule of immunologically active protein (table 1). Modified Mb is stable. No loss of -pppA residues or of the immunological activity was observed in one year of storage at −4°C.

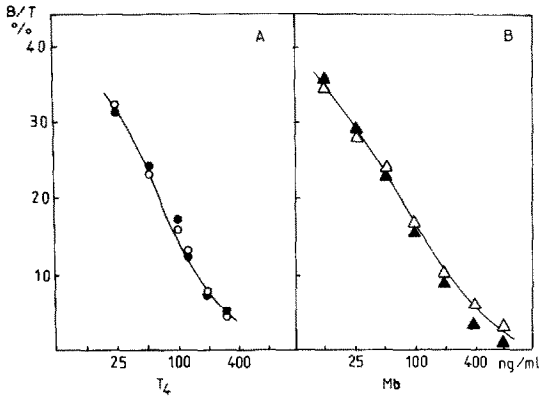

Fig.1. Comparison of the immunological properties of ^{125}I- and ATP-labelled antigens: (A) Displacement of ^{125}I-T_4 from an immune complex by T_4 (⊙) and by MeT_4-ATP (●); (B) displacement of ^{125}I-Mb from an immune complex by Mb (△) and by Mb-ATP (▲). The data were obtained by the RIA procedure where either Mb and T_4, or Mb-ATP and MeT_4-ATP were taken as 'standards': abscissae, concentrations of displacing reagents in samples; ordinates, ratios of the amounts of bound radioactive antigens to the amounts of radioactive antigens in reaction mixtures, B/T (%).

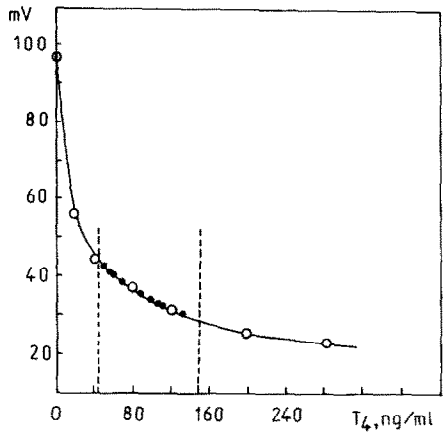

Fig.2. ATP-metric immunoassay of normal sera for T_4: (⊙) standard curve; (●) sera of normal donors; (– – –) borderlines of normal T_4 concentration.

The antigenic properties of MeT_4 and Mb were not changed significantly after the 'labelling' with ATP (fig.1). Fig.2 shows a standard curve and the results of ATP-metric determination of T_4 in a few normal human sera; the curve meets the usual criteria of immunoanalysis; T_4 concentrations found are within the normal range.

Fig.3 shows a standard curve for ATP-metric determination of Mb. With this antigen, direct comparison was made of the ATP-metric assay and the homogeneous assay and the homogeneous RIA method. A good correlation was obtained (r = 0.94). However, the ATP-metric procedure,

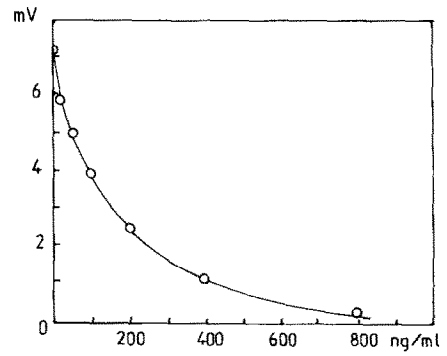

Fig.3. A typical standard curve for solid-phase ATP-metric immunoanalysis for Mb; abscissa, concentration of Mb in sample.

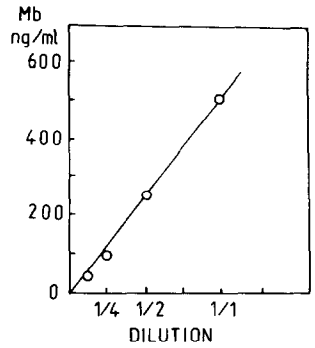

Fig.4. Concentrations of Mb found by ATP-metric immunoanalysis for several dilutions of a human serum with a high content of Mb.

compared with RIA, gave a systematic increase in the concentration of Mb found ($Y = 1.7 \times +31$). The reason for this discrepancy has not been studied, but it is known that solid-phase procedures sometimes give higher values, compared with homogeneous procedures [8]. It is interesting that the non-specific adsorption in the ATP-metric procedure for Mb (fig.3) is zero. The repeatability of the procedure is also satisfactory, the mean square deviation value (σ) was $\pm$ 10%, as calculated from the data of 20 analyses of a single serum (Mb = 28.4 ng/ml). Dilution of a serum with a high content of Mb revealed a linear dependence of the dilution coefficient over Mb 50–500 ng/ml (fig.4). However, the ATP-metric procedure, as well as the standard RIA-procedure

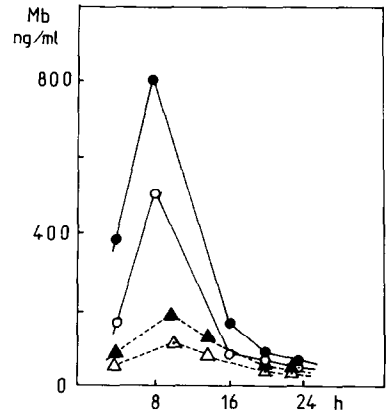

Fig.5. Dynamics of concentrations of Mb in sera of patients with myocardial infarction (⊙,●) and unstable myocardia (▲,▲) found by the RIA-procedure (⊙,▲) and by ATP-metric immunoanalysis (●,▲).

(review [7]), reveals characteristic dynamics of Mb concentrations in sera of patients with myocardial infarction and unstable stenocardia (fig.5); it affords valuable diagnostic information.

4. DISCUSSION

The above data illustrate the validity and the general applicability of the novel ATP-metric immunoanalytical system proposed. The advantages of the procedure, compared with other techniques are: (i) the high stability of the ATP label; (ii) the simplicity of its coupling to antigens; (iii) the high sensitivity of the luminometric procedure; (iv) simplicity of the measuring equipment.

The bioluminescent test with luciferase is not the only ultrosensitive method for ATP detection. Cyclic multi-enzyme systems may be designed in which ATP acts as a catalyst [9]; these systems may use spectrophotometry as the final detection method.

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