Journal of Chromatography, 470 (1989) 185–190
Elsevier Science Publishers B.V., Amsterdam — Printed in The Netherlands

CHROM. 21 269

INVESTIGATIONS ON THE INTERFERON-INDUCED 2′–5′ OLIGOADENYLATE SYSTEM USING ANALYTICAL CAPILLARY ISOTACHOPHORESIS

GERNOT BRUCHELT*
Children's Hospital, Department of Hematology, University of Tuebingen, Ruemelinstrasse 23, D-7400 Tuebingen, (F.R.G.)
MANFRED BUEDENBENDER
Children's Hospital, Department of Surgery, University of Tuebingen, Tuebingen (F.R.G.)
and
JÖRN TREUMER and DIETRICH NIETHAMMER
Children's Hospital, Department of Hematology, University of Tuebingen, Tuebingen (F.R.G.)
and
KARLHEINZ SCHMIDT
Children's Hospital, Department of Surgery, University of Tuebingen, Tuebingen (F.R.G.)

SUMMARY

The activity of the interferon inducible enzyme 2′–5′ oligoadenylate synthetase (2–5A synthetase, E.C. 2.7.7) which converts ATP into a series of 2′–5′ oligoadenylates was measured using analytical capillary isotachophoresis. The turnover rate of ATP during the reaction was monitored by determination of its concentration at the beginning and the end of the 2–5A synthetase reaction. The enzyme was analysed in extracts of peripheral blood mononuclear cells either pretreated or not with interferon.

INTRODUCTION

Interferons are (glyco)proteins with antiviral, antiproliferative and immunoregulatory effects[1]. After interaction with cell surface receptors, the interferons cause several metabolic changes in the cells including the activation of the 2–5A system[2]: an enzyme, the 2–5A synthetase, is produced *de novo*, converting ATP into a series of 2′–5′ oligoadenylates:

$$n\ \mathrm{ATP} \rightarrow \mathrm{ppp5'A2'(p5'A)}_{n-1} + (n-1)\mathrm{PP} \qquad (n = 2\text{–}14)$$

These oligoadenylates activate an endoribonuclease (RNaseL) which splits mRNA, finally resulting in inhibition of protein synthesis. The determination of 2–5A synthetase in mononuclear blood cells has been used clinically as a response parameter during therapy with interferon[3]. Most methods described in the literature use assays with radioactively labelled compounds. Here we describe a non-radioactive test system using analytical capillary isotachophoresis.

0021-9673/89/$03.50

EXPERIMENTAL

Preparation of cell lysates

Peripheral blood mononuclear cells (PBMNCs) were isolated using lymphoprep gradient centrifugation. The cells were cultivated at 37°C for 18 h either in the absence or presence of interferon-beta (Fiblaferon; Bioferon, Laupheim, F.R.G.); 1000 U/ml cell suspension ($2 \cdot 10^6$ cells/ml). Cell lysis was carried out using a buffer solution containing 0.5% Nonidet-P 40.

2–5A synthetase assay

A 20–50 μl volume of cell lysates ($2 \cdot 10^6$ cells per 100 μl) was incubated with Poly(IC)-agarose beads (Pharmacia, Freiburg, F.R.G.) for isolation of the 2–5A synthetase. Thereafter, the beads were added to 25 μl incubation buffer containing ATP (5 m*M*) and creatine phosphate (10 m*M*)/creatine phosphokinase as an ATP-regenerating system. The incubation was carried out at 30°C for up to 20 h. Aliquots of the reaction mixture were withdrawn for isotachophoretic analysis at the beginning and the end of the incubation period (diluted 1:10 in water). A more detailed description of the incubation conditions is given in ref. 4.

Isotachophoresis

The isotachophoretic analysis was carried out on an LKB Tachophor 2127 equipped with an UV (254 nm) and conductivity detector. Current during signal registration: 75 μA. Analyses were carried out at 15°C. Leading electrolyte: 0.01 *M* HCl–β-alanine, pH 3.65 + 0.3% (w/v) methylcellulose. Terminating electrolyte: 0.01 *M* caproic acid. Usually, 6.7-μl samples from the reaction mixtures were injected diluted (usually 1:10) into the Tachophor. Chart speed during detection: 10 cm/min. Capillary: 23 cm × 0.5 mm I.D.

Conversion of ATP into ADP

ATP-containing samples (1–5 mmol/l) were incubated with a solution of glucose (10 m*M*) and hexokinase (10 U/ml) for 1 h at 37°C. Under these conditions, ATP is quantitatively converted into ADP. Isotachophoresis was carried out after dilution of samples (1:10).

RESULTS AND DISCUSSION

Isotachopherogram of ATP in the incubation mixture

In contrast to ADP, AMP and the 2′–5′-oligoadenylates, ATP did not yield a sharp rectangular signal when dissolved in the incubation mixture for the 2–5A synthetase reaction. The reason is not clear; using phosphate-buffered saline, neither the presence nor absence of Mg^{2+} and Ca^{2+} nor of EDTA influence this ATP signal. Rectangular ATP signals were observed only in aqueous solution (Fig. 1a). Therefore, it was advantageous to convert the reaction mixture into ADP for a more accurate determination of the ATP concentration at the beginning and the end of the 2–5A synthetase reaction. This was done using the glucose–hexokinase reaction. Under these conditions, evaluation was possible even at small ATP-turnover rates during the 2–5A synthetase reaction (Fig. 1b). Fig. 2 shows the calibration graph for

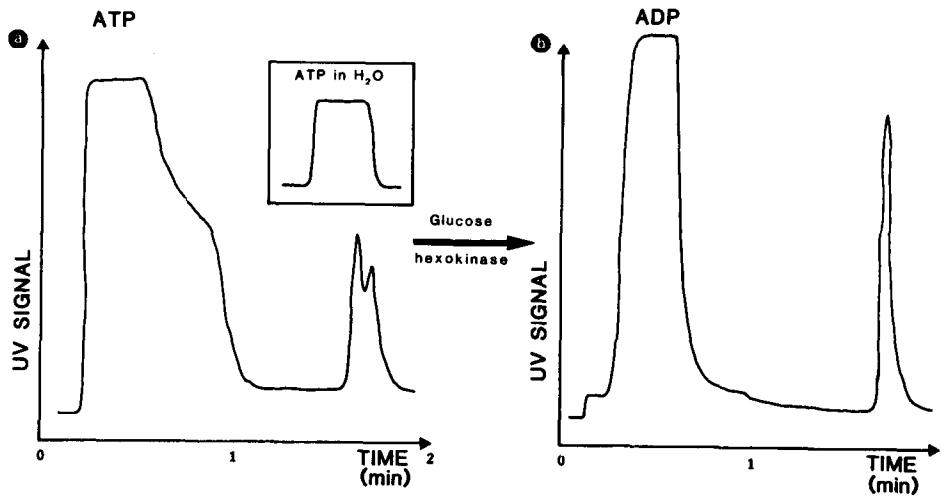

Fig. 1. Isotachopherograms of (a) ATP dissolved in 2–5A synthetase buffer and (b) the same ATP solution in 2–5A synthetase buffer after incubation with glucose–hexokinase.

ATP obtained by measuring the zone lengths of ADP after the glucose–hexokinase reaction.

Changes of the ATP concentration during the 2–5A synthetase reaction

For determination of the 2–5A synthetase activity, the decrease in ATP concentration during the incubation period (up to 20 h) was measured. Because of this long incubation time it was necessary to measure potential non-specific ATP decomposition: Fig. 3a,b shows that non-specific destruction does not occur, probably because of the high efficacy of the ATP-regenerating system, creatine phosphate–creatine phosphokinase, in the incubation mixture. The conversion of ATP into 2'–5' oligoadenylates in the presence of 2–5A synthetase is shown in Fig. 3c. In the example

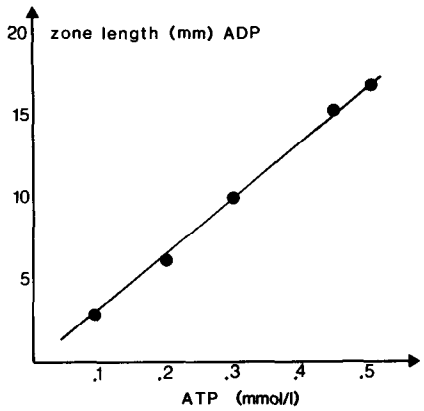

Fig. 2. Calibration graph for different amounts of ATP (1–5 mmol/l) after conversion into ADP using the glucose–hexokinase reaction (samples diluted 1:10 prior to isotachophoresis).

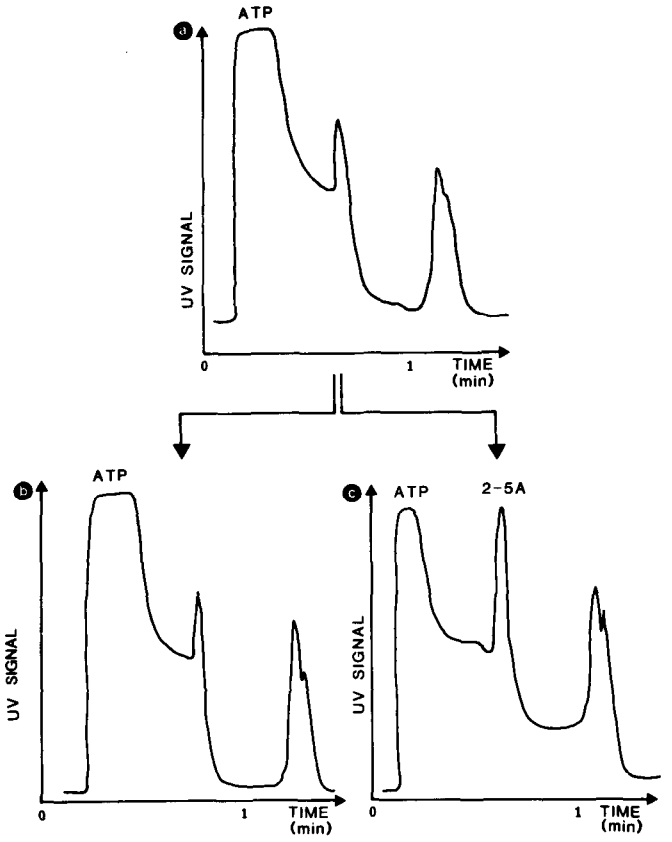

Fig. 3. Isotachopherograms of the 2–5A synthetase reaction. (a) At the start of the reaction; (b) after incubation for 20 h without 2–5A synthetase (lysis buffer instead of cell lysates was used); (c) after incubation for 20 h, in the presence of 2–5A synthetase lysate of $1 \cdot 10^6$ cells incubated with Poly(IC)-agarose beads.

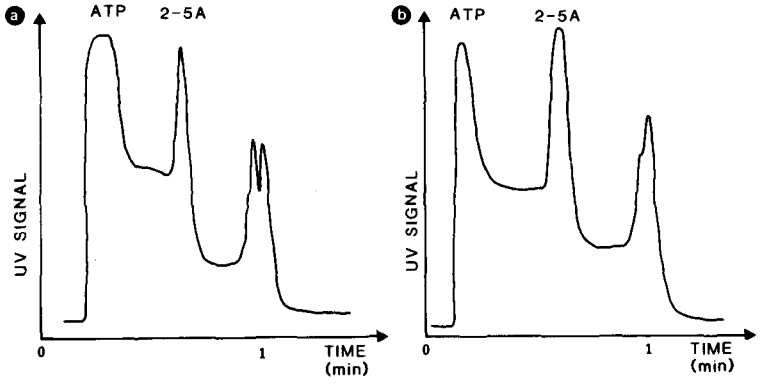

Fig. 4. Isotachopherograms after incubation for 20 h using Poly(IC)-agarose beads loaded with extracts of $5 \cdot 10^5$ PBMNC, (a) not pretreated with interferon, (b) treated with interferon-beta (1000 U/ml cell suspension, incubation time 18 h at 37°C).

given the turnover of ATP during a 20-h incubation period caused by 2–5A synthetase present in the lysate of $1 \cdot 10^6$ PBMNC, is depicted: 50.7% of the amount of ATP present at the beginning of the reaction were transformed to 2'–5' oligoadenylates; therefore, the activity of this cell lysate can be given as the consumption of 3.15 nmol ATP per hour per 10^6 cells.

Influence of pretreatment of PBMNC with interferon on the 2–5A synthetase activity

Fig. 4 shows the ATP signal after incubation for 20 h using 2–5A synthetase isolated from 20-μl lysates of PBMNCs compared with the signal obtained from 20-μl lysates of PBMNCs that were pretreated with 1000 U interferon-beta for 18 h. Simultaneously with the decline of the ATP signal, the signals of oligoadenylates increase. The ATP turnover rate using the extracts from cells pretreated with interferon was 8.75 nmol ATP per hour per 10^6 cells, and that in lysates of PBMNCs not pretreated with interferon was 4.46 nmol ATP per hour per 10^6 cells; *i.e.*, treatment with interferon led to a 1.96-fold increase in 2–5A synthetase activity.

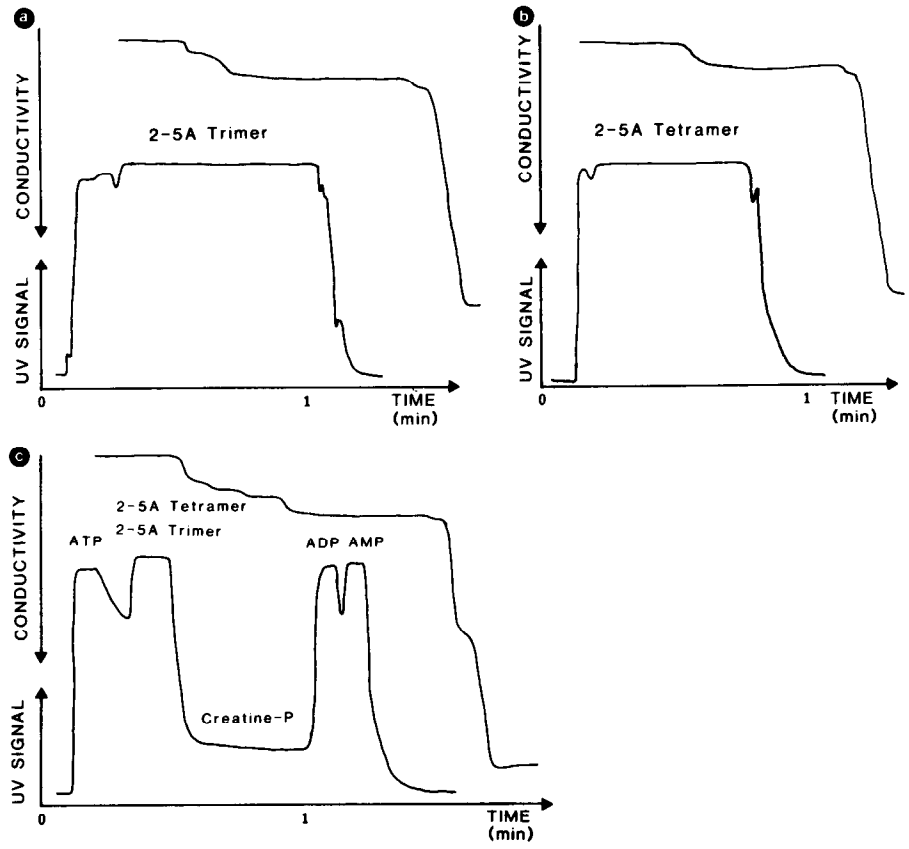

Fig. 5. Isotachopherograms of commercially available 2'–5' oligoadenylates. (a) 2–5A trimer (pppA2'p5'A2'p5'A), 2.3 mmol/l; (b) 2–5A tetramer (pppA2'p5'A2'p5'A2'p5'A), 2.3 mmol/l; (c) tachogram of a mixture consisting of ATP (0.4 mmol/l), ADP (0.4 mmol/l), AMP (0.4 mmol/l), 2–5A trimer (0.17 mmol/l), 2–5A tetramer (0.17 mmol/l) and creatine phosphate (1.0 mmol/l); sample volume 6.7 μl.

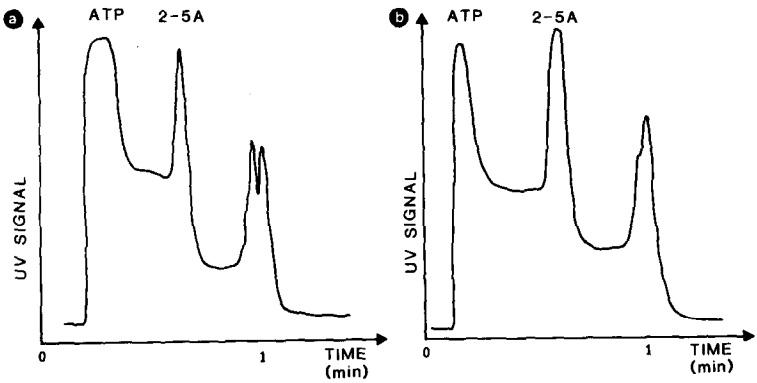

Fig. 6. Characterization of the isotachopherogram of the 2–5A synthetase reaction. (a) After incubation for 20 h; (b) after addition of 2–5A trimer [1 μl 2–5A trimer (50 nmol) + 9 μl reaction mixture].

Isotachophoretic characterization of 2′–5′ oligoadenylates

Fig. 5a shows the isotachopherogram of pppA2′p5′A2′p5′A (2–5A trimer), Fig. 5b that of the 2–5A tetramer (pppA2′p5′A2′p5′A2′p5′A). Both commercially available compounds contain impurities. The net mobilities of the trimeric and tetrameric forms representing the major fraction of oligoadenylates formed during the 2–5A synthetase reaction are very similar, therefore they cannot be separated from each other by isotachophoresis using the system described here. This is demonstrated in Fig. 5c: in a synthetic mixture of ATP, ADP, AMP, creatine phosphate, 2–5A trimer and 2–5A tetramer only the two oligoadenylates were not separated. Separation was also not possible in the presence of spacer molecules (Ampholine carrier ampholytes, LKB; pH 3.5–10; data not shown). However, for the estimation of the activity of 2–5A synthetase, this fact is not relevant because it is more advantageous to determine the decrease in ATP instead of the heterogeneous population of 2–5 oligoadenylates that is formed during the 2–5A synthetase reaction. Although a series of different oligoadenylates are formed, preferentially the trimeric and the tetrameric forms, only one additional peak appears in the isotachophoregram. Evidence that this second peak consists of 2–5 oligoadenylates formed during the 2–5A synthetase reaction was obtained by substitution of 2–5A as shown in Fig. 6: after addition of 2–5A trimer to the 2–5A synthetase reaction mixture after incubation for 20 h, no additional peak appeared, but the zone length of the second peak increased.

In conclusion, analytical capillary isotachophoresis can be successfully used for determination of 2–5A synthetase activity, especially when small numbers of samples are to be analysed. The determination of nucleotides is highly reproducible, therefore also very small turnover rates of enzymatic reactions can be measured accurately. This is especially advantageous in the case of the 2–5A synthetase reaction because it is difficult to perform the assay under ATP-saturated conditions[4].

REFERENCES

1 S. Pestka, J. A. Langer, K. C. Zoon and Ch. E. Samuel, *Annu. Rev. Biochem.*, 56 (1987) 727–777.
2 M. I. Johnston and P. F. Torrence, in R. M. Friedmann (Editor), *Interferon*, Vol. 3, Elsevier, Amsterdam, New York, Oxford, 1984, Ch. 7.
3 J. A. Merritt, D. M. Meltzer, L. A. Ball and E. C. Borden, *Prog. Clin. Biol. Res.*, 202 (1985) 423–430.
4 G. Bruchelt, J. Beck, K. Schilbach-Stückle, E. Koscielniak, J. Treuner and D. Niethammer, *J. Clin. Chem. Clin. Biochem.*, 25 (1987) 879–888.