YEAST HEXOKINASE REACTION WITH ADENOSINE 5'-*O*-TRIPHOSPHATE AND ADENOSINE 5'-*O*-(1-THIO-TRIPHOSPHATE) MONITORED BY LIQUID CHROMATOGRAPHY

Kurt-Wilhelm STAHL and Eckhard SCHLIMME
Institut für Klinische Biochemie und Physiologische Chemie, Medizinische Hochschule Hannover, D-3000 Hannover, BRD

and

Fritz ECKSTEIN
Max-Planck-Institut für Experimentelle Medizin, Abt. Chemie D-3400 Göttingen, BRD

Received 19 December 1973
Revised version received 23 January 1974
Original figures received 14 February 1974

1. Introduction

Yeast hexokinase has been extensively studied but the mechanism of the enzymatic reaction is still a subject of lively discussion [1, 2 and cited ref.]. Systematically modified ANP* seem to be useful probes for better understanding of the hexokinase nucleotide interactions in binding and catalysis processes. In previous studies of this kind coupled indicator reactions were used [2, 3]. However, direct monitoring of the nucleotide turnover is the best guarantee of true measurements and exact interpretations of results.

Therefore, we should like to introduce high efficiency LC into enzymology [4, 5] and to demonstrate the application of this direct analytical method in studies with ANP-thiophosphate analogues [6, 7] and hexokinase. Substitution of oxygen by sulfur in the α- and β-position of the phosphate moiety of the nucleotide may lead to diaisostereomeric forms [6, 8]. Using an arbitrary nomenclature, peak 4 in the chromatogram in fig. 1 corresponds to the so called A-form of ADP (αS). Consequently, the diastereoisomer, peak 5, is the B-form.

LC is apparently the only analytical method described [4] for separating the diastereoisomeric forms of ADP (αS). In these experiments, LC enables us to monitor the reaction of hexokinase with the isomeric mixture of the A- and B-forms of ATP (αS) in one incubation assay and both product forms, ADP (αS) A-form and ADP (αS) B-form can be determined in a single analysis.

* Abbreviations: ANP, adenine nucleotide(s); LC, liquid chromatography; ADP (αS), adenosine 5'-*O*-(1-thio-disphosphate); ATP (αS), adenosin 5'-*O*-(1-thio-triphosphate).

2. Materials and methods

2.1. *Materials*

Hexokinase {ATP: D-hexose-6-phospho-transferase; EC 2.7.1.1; 2 mg/ml; 140 IU/mg} was purchased from Boehringer Biochemica, Mannheim, GFR. The thio-phosphate analogues of ANP were synthesized as described earlier [6, 7]. All other substances were of reagent grade and are commonly available.

2.2. *Enzyme assay*

The incubation medium which was optimum for ATP in phosphoryltransfer reactions with hexokinase

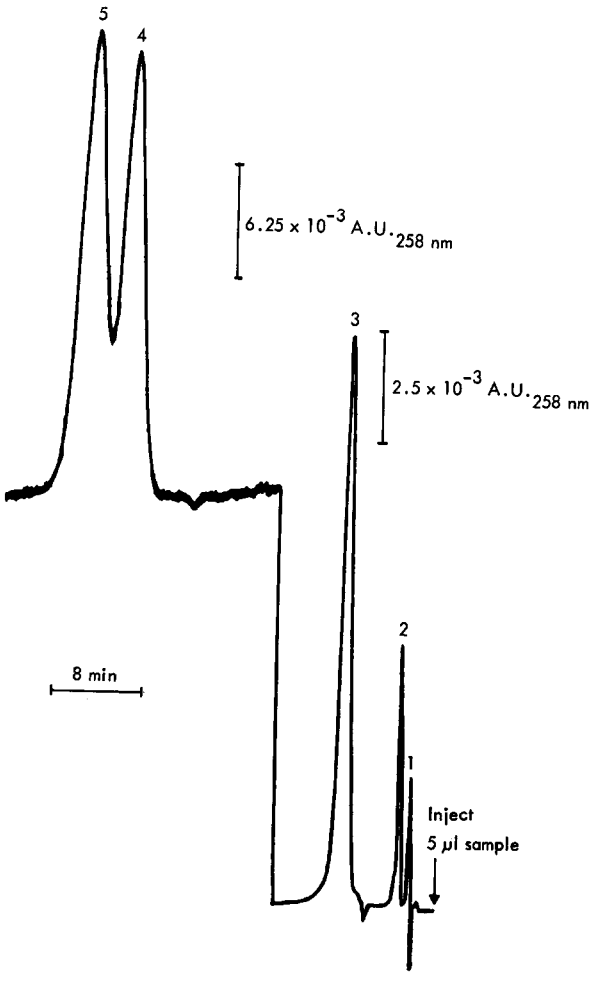

Fig. 1. LC separation [4, 9] of a standard mixture of AMP, ADP and their α-thiophosphate analogues on a 300 mm glass column filled with anion exchanger IONEX SB 0520 purchased from Macherey Nagel + Co Düren GFR. The differences in the base line level are due to a change in sensitivity. 1 = AMP; 2 = AMP (αS); 3 = ADP; 4 = ADP (αS) A-form; 5 = ADP (αS) B-form.

contained: 40 mM triethanolamine, 8 mM $MgCl_2$; 40 mM glucose and 2 µl hexokinase (in a total volume of 310 µl) at pH 7.8, and 25°C. The concentration range of nucleoside triphosphates was from 0.061 to 4.98 mM for ATP and from 0.31 to 3.79 mM for ATP (αS) consisting of 42.52% of the A- and 55.87% of the B-diastereoisomer as previously determined by LC [4, 9]. The reactions were started by addition of hexokinase.

Hexokinase was tested for ATPase activity [10] by substitution of mannitol for glucose under the conditions described above.

2.3. *LC analysis*

Anion-exchange LC of ANP and of diastereoisomeric thio-phosphate analogues was performed as described elsewhere [4, 9]. The chromatogram of a standard mixture is shown in fig. 1. Special care was taken to keep all parameters of the LC system constant, e.g. composition and linear velocity of the mobile phase and column temperature. Peak height under these conditions was a reproducible quantitative measure with sufficient accuracy, as can be seen from the standard curve of measurement of ADP (see fig. 2). Each point represents the arithmetical mean of several 5 µl samples injected on the column at different time intervals throughout the kinetic measurements. The maximum deviation of the mean for the 95% range of confidence was ± 0.7% as obtained in calibrations of ADP and ADP (αS) solutions in a concentration range of 0.018 to 0.34 mM.

2.4. *Sampling technique*

Without coupled glucose-6-phosphate dehydrogenase reaction, hexokinase reaction was found linear for at least 16 sec at the highest ATP concentration used in the incubation assays. Therefore, within this period four or five 20 µl samples (see fig. 3) were taken from the incubation assays and denatured at different time intervals in 10 µl of 0.16 N $HClO_4$ which contained 2×10^{-4} M 5′ AMP as an internal standard σ_1. This short period required an assistant for each sample as it was taken and denatured. The beginning and end of the incubations were automatically registered by an event marker on a paper strip recorder running at a constant chart speed of 250 mm/min. Immediately afterwards, the samples were frozen and stored in N_2 until further analyzed by LC. Tracer amounts of uniformly ^{14}C-labelled 5′ AMP of high specific activity (Radiochemical Centre, Amersham, England) served as an internal standard σ_2 in the incubation assays. Procedural errors were minimized by using the ratio σ_1/σ_2 in the calculations. A similar procedure was used for monitoring the turnover of the thio-phosphate analogues of ATP. Sample timing was less critical

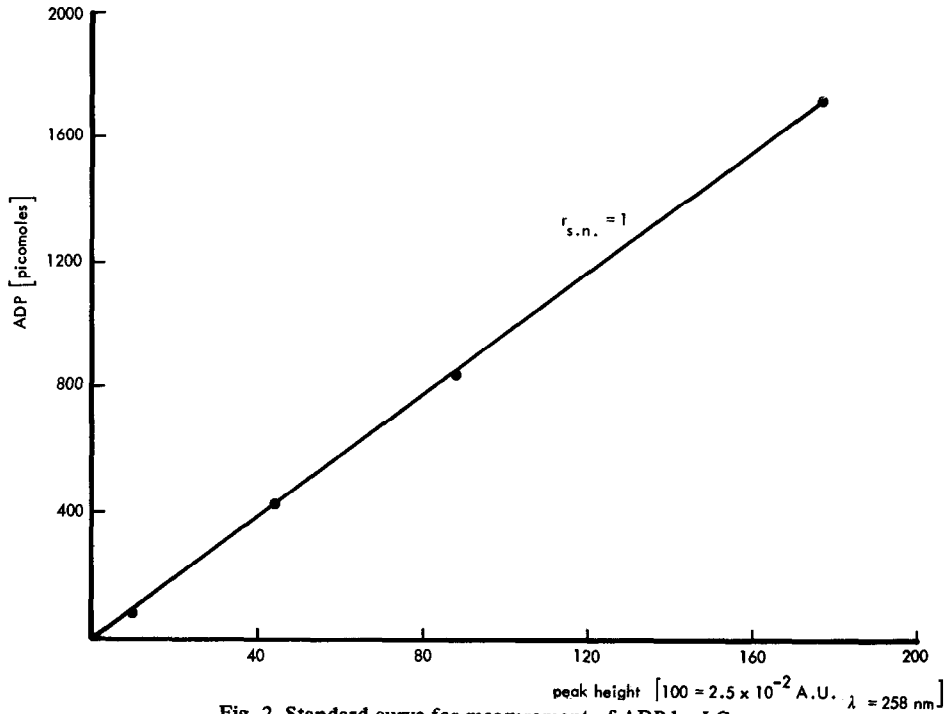

Fig. 2. Standard curve for measurement of ADP by LC.

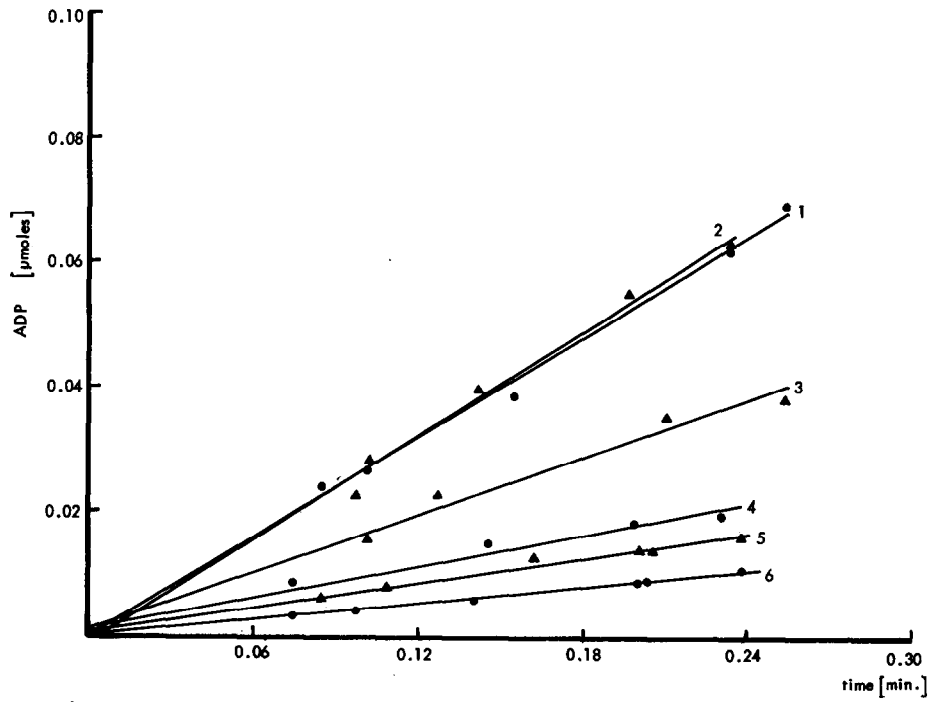

Fig. 3. ADP {μmoles} produced by 4 μg hexokinase (EC 2.7.1.1) at times indicated for different ATP–substrate concentrations 1 = 25.674 X 10^{-4} M; 2 = 5.802 X 10^{-4} M; 3 = 2.990 X 10^{-4} M; 4 = 1.183 X 10^{-4} M; 5 = 1.003 X 10^{-4} M; 6 = 0.609 X 6 = 0.609 X 10^{-4} M.

since the linear range for reaction velocity was 1 min for ATP (αS) A-form and 100 min for ATP (αS) B-form.

3. Results

As can be seen from figs. 4 and 5 K_M- and V_{max} values were derived by plotting $\frac{1}{v}$** with respect to $\frac{1}{S}$ of the nucleoside triphosphates. The RC95% for a and b in the linear function $y=ax+b$ were determined according to common statistical methods. The RC* 95% of K_M is limited by the two quotients $(a+RC95\%)/(b-RC95\%)$ and $(a-RC95\%)/(b+RC95\%)$, that of V_{max} by the reciprocals of $(b+RC95\%)$ and of $(b-RC95\%)$.

** Abbreviations: v, initial velocity; S, substrate concentration; RC95%, 95% ranges of confidence; RC*95%, range of error for graphic determination of K_M and V_{max} in the Lineweaven–Burk plots.

3.1. *ATP*

Initial velocities, which were measured by LC within a maximum time of 16 sec were linear and the origin of the graph in fig. 3 is within the RC95% for all b-values of table 1. Using these initial velocities, the following values were obtained from the Lineweaver–Burk plot (see fig. 4): K_M = 5.0 (4.1 – 6.1; RC*95%) × 10^{-4} M and V_{max} = 105 (90–126; RC*95%) units/mg hexokinase.

Table 1

Linear functions y = $ax + b$ of the initial velocities from fig. 3, RC95% for a and b and linear correlation coefficients for small numbers $\{r_{s.n.}\}$.

1. $r_{s.n.}$ = 0.999	$y = (0.269 \pm 0.016) X - (0.00001 \pm 0.00267)$
2. $r_{s.n.}$ = 0.998	$y = (0.279 \pm 0.026) X - (0.00096 \pm 0.00383)$
3. $r_{s.n.}$ = 0.996	$y = (0.156 \pm 0.020) X + (0.00096 \pm 0.00302)$
4. $r_{s.n.}$ = 0.993	$y = (0.085 \pm 0.019) X + (0.00131 \pm 0.00287)$
5. $r_{s.n.}$ = 0.993	$y = (0.067 \pm 0.011) X + (0.00067 \pm 0.00171)$
6. $r_{s.n.}$ = 0.999	$y = (0.045 \pm 0.002) X + (0.00014 \pm 0.00031)$

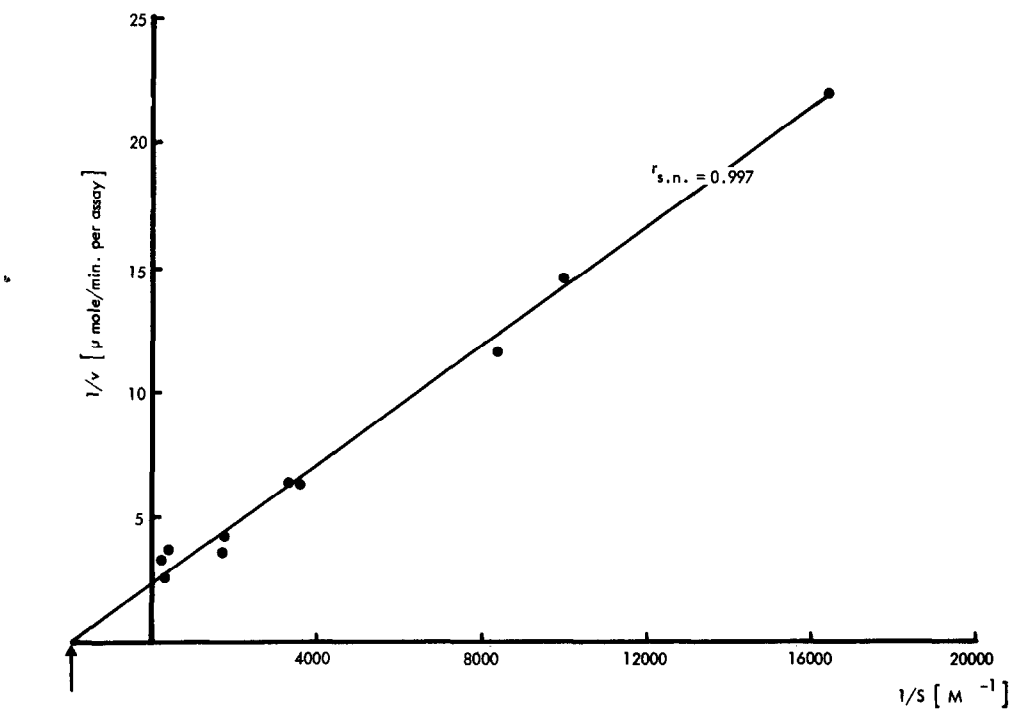

Fig. 4. Determination of K_M (5 × 10^{-4} M) and V_{max} (105 units/mg) for ATP in hexokinase (EC 2.7.1.1) reaction. The experimental points were derived from two different incubation assays.

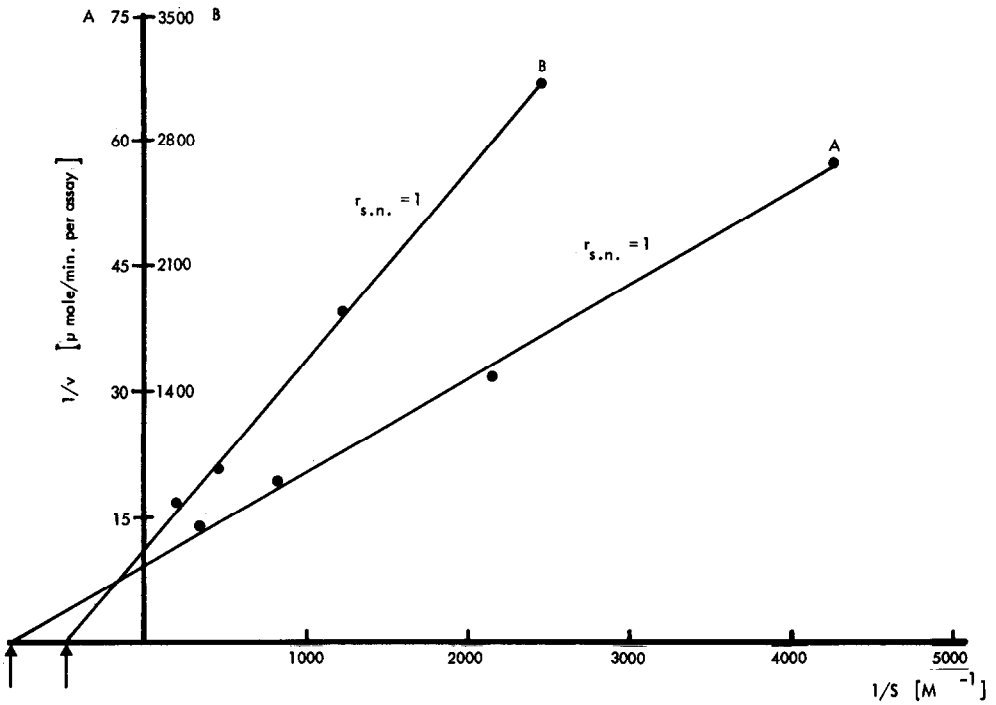

Fig. 5. Determination of K'_M ATP (αS) A-form (1.2×10^{-3} M), V'_{max} ATP (αS) A-form (27 units/mg), K'_M ATP (αS) B-form ($2.02 \cdot 10^{-3}$) and V'_{max} ATP (αS) B-form (0.48 units/mg) in the isomeric mixture of ATP (αS) in hexokinase (E.C. 2.7.1.1) reaction. A and B represent the different scales chosen for the A- and B-forms of ATP (αS) on the ordinate.

3.2. *ATP (αS)*

In the same way the following values were obtained for ATP (αS) A-form: K'_M = 1.2 (1.17 – 1.23; RC*95%) × 10^{-3} M and V'_{max} = 27 (± 0.5; RC*95%) units/mg hexokinase, i.e. 28.4% $V_{max\ ATP}$ and for ATP (αS) B-form: K'_M = 2.02 (1.99–2.05; RC*95%) × 10^{-3} M and V'_{max} = 0.48 (± 0.01; RC*95%) using/mg hexokinase, i.e. 0.5% $V_{max\ ATP}$ (see fig. 5). Our experimental values are designated K'_M and V'_{max} in order to differentiate them from the values which would have resulted from separate incubations of ATP (αS) A-form and ATP (αS) B-form.

4. Discussion

The K_M of ATP determined by LC differs only slightly from published values. This difference cannot be attributed to ATPase activity [10], which was not detectable in hexokinase within the sampling time, even at high ATP concentrations (see Materials and methods, 2.2.).

Chemical synthesis of ATP (αS) [6, 8] always leads to a mixture of the two diastereoisomeric forms A and B, which was used as a substrate for the hexokinase assays (see Materials and methods, 2.2.). Since the A- and B-forms react almost consecutively (see Materials and methods, 2.4.), the kinetic properties of ATP (αS) A-form are measured in the presence of equimolar amounts of ATP (αS) B-form, whereas the B-form of ATP (αS) reacts in the presence of equimolar amounts of diphosphate resulting from reaction of ATP (αS) A-form. ATP (αS) B-form and ADP (αS) A-form are likely to have inhibitory effects on the turnover over the two forms of the triphosphates. The inhibitor/substrate ratio may be considered as constant for all measured ATP (αS) concentrations. The Lineweaver–Burk plots resulting from separate incubations of the diastereoisomeric triphosphates would parallel the experimental points (see fig. 5). Our values ($1/K'_M$ and $1/V'_{max}$)

are higher than the true values ($1/K_M$ and $1/V_{max}$) for both isomers and K_M/K'_M is equal to V_{max}/V'_{max}.

From these data, the following conclusions can be drawn:

1. Substitution of an oxygen atom by sulfur in the phosphate chain seems to affect the binding affinities of the adenosine triphosphates as seen by the differences of the K_M for ATP and the isomers of ATP (αS).

2. On the other hand, catalysis is rather sensitive to conformational changes in the phosphate moiety of the ATP molecule: V'_{max} of the B-form is only 2% of the V'_{max} of the A-form. From the data presented here the ATP (αS) A-form appears to be more similar to ATP in the enzyme–substrate complex since $V'_{max\ ATP\ (\alpha S)\ A\text{-}form}$ is 28% of $V_{max\ ATP}$. It is interesting to note that substitution of an oxygen atom by sulfur in the γ-phosphate of ATP as in ATP (γS) [7] leads to complete loss of substrate activity in the hexokinase reaction [11].

3. This difference in enzymatic activity can be used for enzymatic enrichment which could lead to purification of the diastereoisomers on a preparative scale [8].

Modifications in the phosphate moiety of adenine nucleotides, especially those leading to chiral centres, appear to be promising tools for studies of phosphoryl-transfer reactions.

Acknowledgements

We are grateful to Professor Dr Walther Lamprecht for genereous support; to A. Baranowski, E. Schiller and H. Schink for their technical assistance and to Mrs S. Vančura for kindly reading the manuscript.

This work was partially sponsored by the Deutsche Forschungsgemeinschaft.

References

[1] Rudolph, F. B. and Fromm, H. J. (1971) J. Biol. Chem. 246, 6611.
[2] Hohnadel, D. C. and Cooper, C. (1972) Eur. J. Biochem. 31, 180.
[3] Hampton, A., Howgate, P., Harper, P. J., Perini, F., Kappler, F. and Preston, R. K. (1973) Biochemistry 12, 3328.
[4] Stahl, K.-W., Schlimme, E. and Bojanowski, D. (1973) J. Chromatog. 83, 395.
[5] Stahl, K.-W., Schlimme, E. and Lamprecht, W. (1973) Abstr. Kongress der Deutschen Gesellschaft für Klinische Chemie Hannover, GFR
[6] Eckstein, F. and Gindl, H. (1970) Eur. J. Biochem. 13, 558.
[7] Goody, R. S. and Eckstein, F. (1971) J. Amer. Chem. Soc. 93, 6252.
[8] Eckstein, F. and Goody, R. S., manuscript in preparation.
[9] Stahl, K.-W., Eckstein, F. and Schlimme, E., manuscript in preparation.
[10] Rudolph, F. B. and Fromm, H. J. (1971) J. Biol. Chem. 246, 2104.
[11] Schlimme, E., Lamprecht, W., Eckstein, F. and Goody, R. S. (1973) Eur. J. Biochem. 40, 485.