Biochimica et Biophysica Acta, 615 (1980) 85—93

BBA 69094

A HEXOKINASE FROM FISH LIVER WITH WIDE SPECIFICITY FOR NUCLEOTIDES AS PHOSPHORYL DONOR

FUMIO NAGAYAMA, HIROSHI OHSHIMA, HIROKAZU SUZUKI and TOSHIAKI OHSHIMA

Department of Food Science and Technology, Tokyo University of Fisheries, Konan-4, Minato-ku, Tokyo 108 (Japan)

(Received January 3rd, 1980)

Key words: ADP; Hexokinase; Nucleotide; Phosphoryl donor; (Fish liver, Rainbow trout)

Summary

The liver of rainbow trout contains two hexokinases (ATP:D-hexose 6-phosphotransferase, EC 2.7.1.1) designated C and D from the elution pattern in DEAE-cellulose column chromatography. Hexokinase D has been purified about 50-fold from the liver of rainbow trout by chromatography with DEAE-cellulose and Sephadex G-200, and by isoelectric focusing. The properties of hexokinase D were similar to those of mammalian hexokinase III with respect to the K_m values for ATP and glucose and the substrate inhibition by glucose at high concentration. However, the enzyme showed a wide specificity for nucleotides as the phosphoryl donor. Although it has been reported that the only effective nucleotide as the phosphoryl donor for hexokinase from various origin is ATP, and that ADP, a reaction product, inhibits the enzyme, hexokinase D from the rainbow-trout liver was found to be able to form glucose 6-phosphate (Glc-6-*P*) from glucose and various nucleotides such as ATP, ADP, CTP, GTP, UTP and UDP. The reaction products from ADP and glucose, Glc-6-*P* and AMP, were identified by chromatography on ion-exchange resin column and paper. The enzyme D was not inhibited by ADP but was strongly inhibited by AMP, which is a reaction product from ADP.

Introduction

Recent work from this laboratory has indicated that there are four types of hexokinase (ATP:D-hexose 6-phosphotransferase, EC 2.7.1.1) in fish liver, which were designated tentatively hexokinases A, B, C and D from the elution pattern in DEAE-cellulose column chromatography [1]. The enzymes were

partially purified from the liver extracts of six different fish species, and their kinetic and other properties were investigated. The composition of hexokinases in fish liver seemed to vary, dependent on fish species. From the elution pattern in DEAE-cellulose column chromatography and the apparent K_m values for ATP and glucose, fish hexokinases A and D resembled mammalian hexokinases I and III, respectively, while hexokinases B and C resembled mammalian enzyme II. Activity of glucokinase (ATP:D-glucose 6-phosphotransferase, EC 2.7.1.2), which is called hexokinase IV, was not detected in fish liver. However, an interesting discrepancy in the properties between fish and mammalian hexokinases was seen on hexokinase D from the rainbow trout and yellowtail which was not inhibited by ADP. Furthermore, when ATP was replaced by ADP in the assay system of hexokinase D coupled with Glc-6-*P* dehydrogenase and NADP, there was an apparent increase in the absorbance at 340 nm resulting from NADP reduction, indicating the formation of Glc-6-*P* in the system. This phenomenon suggests that fish hexokinase D is capable of utilizing ADP as a phosphoryl donor, although it is known that the only effective phosphoryl donor for the hexokinase reaction is ATP [2], and that the reaction products, ADP and Glc-6-*P*, inhibit the enzyme [2,3].

In this paper, we will present the evidence of utilization of ADP as a phosphoryl donor by hexokinase D from the liver of rainbow trout, based on the kinetic investigation and analysis of the reaction products.

Materials and Methods

Reagents. Nucleotides and Glc-6-*P* dehydrogenase (yeast, Grade I) were obtained from Boehringer Mannheim GmbH Biochemica, Mannheim; myokinase (rabbit muscle, Grade III) from Sigma Chemical Co., St. Louis, MO; Sephadex from Pharmacia Fine Chemicals, Uppsala, and DEAE-cellulose from Brown Co., Berlin. All other reagents of analytical grade were obtained from Wako Pure Chemical Industries, Ltd., Osaka.

Fish. Male rainbow trout, *Salmo gairdnerii*, weighing from 200 to 500 g, were supplied from Akashina Trout Farm, Nagano.

Preparation of hexokinase. The procedure was essentially that of Grossbard and Schimke [2] with minor modification. About 30 g of the trout liver was homogenized with a Potter-Elvehjem homogenizer in an equal volume of 10 mM potassium phosphate buffer, pH 7.0, containing 1 mM EDTA and 4 mM *N*-acetyl cysteine. The homogenate was centrifuged at 105 000 × *g* for 60 min. The resulting supernatant solution was subjected to further purification processes using Sephadex G-25, DEAE-cellulose and Sephadex G-200 column chromatography. The isoelectric focusing was performed with LKB 8101 Ampholine Electrofocusing Equipment.

Determination of hexokinase activity. The activity of hexokinase was measured spectrophotometrically in an assay system coupled with Glc-6-*P* dehydrogenase according to the method of Shatton et al. [4]. The reaction mixture contained in a final volume of 0.6 ml: 75 mM Tris-HCl buffer (pH 7.5), 50 μM glucose, 7.5 mM $MgCl_2$, 0.5 mM NADP, 0.3 IU of Glc-6-*P* dehydrogenase, and 2.5 mM ATP or other nucleotides as phosphoryl donor at the concentration as described in the text. The reaction was started by adding 0.1 ml of the hexo-

kinase preparation. Initial reaction rate was measured by monitoring the increase of absorbance at 340 nm with a JASCO Spectrophotometer UVIDEC 2DW equipped with a recorder. One enzyme unit was defined as the amount of enzyme catalyzing the formation of 1 μmol of Glc-6-*P* per min at 25°C.

Identification of the reaction products. In addition to the enzymic analysis, Glc-6-*P* was identified by paper chromatography according to the method of Bandurski and Axelrod [5].

ATP, ADP and AMP were analyzed by the method of Ehira et al. [6], using Dowex 1-X4 (Cl^-) column chromatography. After the sample solution was applied to the Dowex column (0.6 × 6 cm), the column was washed with 2 ml of distilled water, and nucleotides were eluted with a linear gradient of Cl^-, each using 120 ml $5 \cdot 10^{-4}$ N HCl and 0.1 N HCl containing 0.15 M NaCl at a flow rate of 1 ml per min. Nucleotide concentration was determined by measuring the absorbance of the eluate at 260 nm. The nucleotides were also identified by paper chromatography [7].

Results

Purification and some physical properties of hexokinase D from the rainbow-trout liver

A typical elution pattern of hexokinase activity from a DEAE-cellulose column is shown in Fig. 1. The enzyme activity could be separated into two components, hexokinases C and D. The activity composition of hexokinases C and D in the rainbow-trout liver was 6 : 4. Although we have reported previously that the rainbow-trout liver contained only hexokinase D [1], the pattern obtained here, showing the activities of two hexokinases, is similar to the enzyme

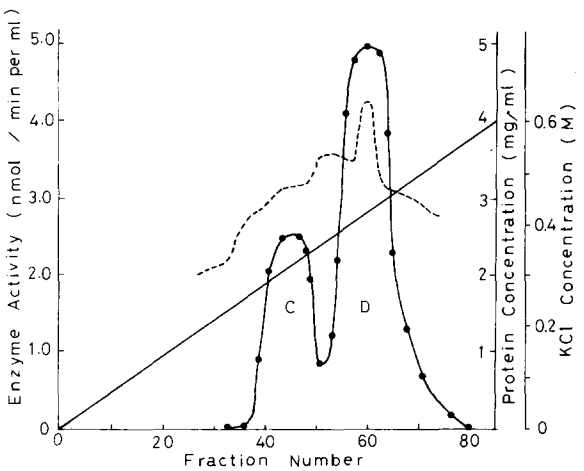

Fig. 1. DEAE-cellulose column chromatography of rainbow-trout liver hexokinase. The liver was extracted with 10 mM potassium phosphate buffer, pH 7.5, containing 1 mM EDTA and 4 mM *N*-acetyl cysteine. The extract was centrifuted at 105 000 × *g* for 60 min, and the resulting supernatant was passed through a Sephadex G-25 column (3 × 30 cm). The enzyme fraction was applied onto a DEAE-cellulose column (2 × 20 cm), and the enzyme was eluted with 400 ml of a linear gradient of KCl from 0 to 0.6 M in 10 mM phosphate buffer. 4-ml fractions were collected. ●———●, hexokinase activity; - - - - -, protein concentration, mg/ml.

pattern of the yellowtail liver. The discrepancy between these results remains to be solved, but it is probably due to the difference in the physiological condition of the trout examined.

Hexokinase D separated by DEAE-cellulose column chromatography was further purified by $(NH_4)_2SO_4$ fractionation and Sephadex G-200 column chromatography. Fig. 2 shows a typical elution pattern of the enzyme from a Sephadex column. Hexokinase D was purified 50-fold (overall purification) and was found to have a specific activity of about 0.25 units per mg protein at 25°C. Due to the instability, it was difficult to obtain an enzyme preparation with higher specific activity, even if all processes for purification were performed in a cold room.

Fig. 3 shows the pattern of isoelectric focusing of hexokinase D from the rainbow-trout liver. The isoelectric point of the enzyme was found to be pH 5.2.

Specificity of hexokinase D for nucleotides as phosphoryl donor

The enzyme activity in the presence of various nucleotides was assayed by monitoring the formation of Glc-6-*P* as described in Materials and Methods. Hexokinase D was found to utilize not only ATP but other nucleotides as the phosphoryl donor as seen in Table I. Among the nucleotides examined so far, ADP was the most effective phosphoryl donor for the enzyme, and the activity with ADP was 80% higher than that with ATP. It has been reported that the

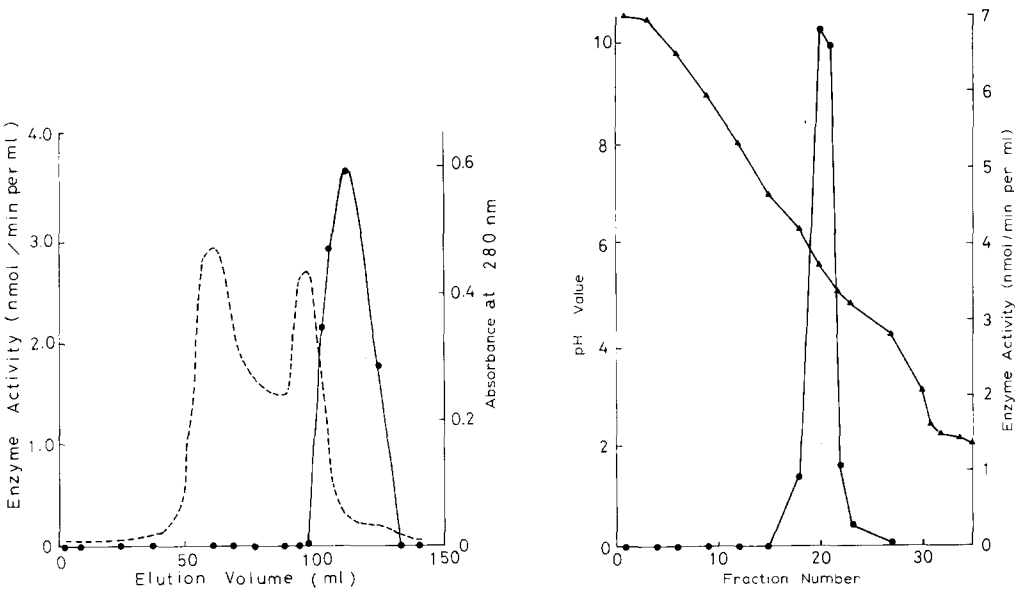

Fig. 2. Gel filtration of hexokinase D of rainbow-trout liver on a Sephadex G-200 column. Hexokinase D eluted from DEAE-cellulose column was treated with $(NH_4)_2SO_4$ and the enzyme (45—80% saturation) was applied onto a Sephadex G-200 column (2.5 × 40 cm). ●———●, hexokinase activity; ------, absorbance at 280 nm.

Fig. 3. Isoelectric focusing of hexokinase D of rainbow-trout liver. ●———●, hexokinase activity; ▲———▲, pH.

TABLE I

SPECIFICITY OF HEXOKINASE D OF RAINBOW-TROUT LIVER FOR NUCLEOTIDES AS PHOSPHORYL DONOR

Nucleotide (2.5 mM)	Relative activity (%)
ATP	100
ADP	179
CTP	71
GTP	60
UTP	37
UDP	39
ITP	9

only effective phosphoryl donor in the hexokinase reaction is ATP [2], and ADP is known to inhibit hexokinase [3]. Such a wide specificity of hexokinase for nucleotides as observed on hexokinase D of the trout liver has not until now been known.

Identification of the reaction products

In order to see whether hexokinase D isolated from the liver of rainbow trout is capable of utilizing ADP as a phosphoryl donor, the reaction products after incubation of the purified enzyme with ADP and glucose were identified by chromatography on ion-exchange resin and paper.

Fig. 4 and Table II show the chromatographic pattern of the nucleotides on a Dowex column and their quantitative composition. We initially checked on a possibility that the enzyme preparation was contaminated with myokinase catalyzing the formation of ATP and AMP from ADP. As seen in Fig. 4, no ATP could be detected after incubating the enzyme with ADP; that is, myokinase was not contained in our enzyme preparation.

When the enzyme was incubated with ADP and glucose, an apparent increase of AMP and a decrease of ADP were observed, whilst such a change did not occur if glucose was omitted from the reaction system. The formation of AMP was also confirmed by paper chromatography.

Another indication that the enzyme utilizes ADP as well as ATP as the phosphoryl donor comes from the analysis of Glc-6-*P* by paper chromatography. Glc-6-*P* was apparently formed from ADP (or ATP) and glucose in the presence of the enzyme. In addition, the Glc-6-*P* formation was confirmed spectrophotometrically in a system coupled with Glc-6-*P* dehydrogenase and NADP; the increase of absorbance at 340 nm resulting from the NADP reduction was observed. Such a change in the absorbance did not occur if one of the components of the reaction system was omitted.

Although the reaction products in the system containing other nucleotides such as CTP were not analyzed, formation of Glc-6-*P* leaves no room for doubt since the change in absorbance was observed with the enzyme assay system coupled with Glc-6-*P* dehydrogenase and NADP.

Kinetic properties of hexokinase

The apparent K_m values for the substrates were calculated from Lineweaver-

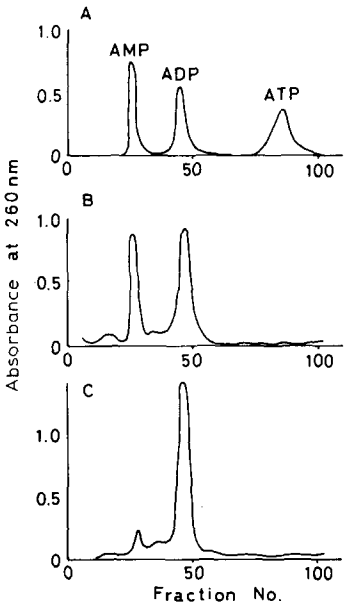

Fig. 4. Separation of nucleotides by ion-exchange chromatography. A. Authentic compounds. Solution containing 2.5 μmol nucleotide was charged on a Dowex 1-X4 (Cl^-) column equilibrated with 0.2 N NH_4OH. The nucleotide was eluted with a linear gradient of Cl^- using each 120 ml of $5 \cdot 10^{-4}$ N HCl and 0.1 N HCl containing 0.15 M NaCl at a flow rate of 1 ml per min. B. Test mixtures. The reaction mixtures consisted of 0.3 ml of purified hexokinase D, 50 μl each of 30 mM ADP and 6 mM glucose, 0.1 ml of 0.45 M Tris-HCl buffer, pH 7.5, 50 μl each of 90 mM $MgCl_2$ and distilled water were incubated at 25°C for 15 h. The reaction mixture was charged on the column and nucleotides were eluted as described above. C. Control mixture. The same reaction mixture as B from which glucose was omitted was incubated at 25°C for 15 h.

Burk plots (Figs. 5 and 6, and Table III). It has been already reported that hexokinase D from the rainbow trout and yellowtail resembles hexokinase III from the rat liver [2] with respect to the substrate (glucose) affinity and inhibition by glucose at high concentration [1]. As seen in the reaction system containing ATP, hexokinase D was found to be also inhibited by glucose at high concentration when ADP was used as a phosphoryl donor (Fig. 5). The K_m value for glucose in the presence of ADP was five times as high as that in the presence of ATP. The K_m value for ADP was about a third of that for ATP.

TABLE II

NUCLEOTIDE COMPOSITION OF THE REACTION MIXTURE AFTER INCUBATION WITH OR WITHOUT HEXOKINASE D AT 25°C FOR 15 H

Constituent	Composition (%)		
	AMP	ADP	ATP
ADP alone	17	83	—
Myokinase + ADP	43	45	12
Hexokinase D + ADP	17	83	—
Hexokinase D + ADP + glucose	35	65	—
Hexokinase D + ATP + glucose	12	43	45

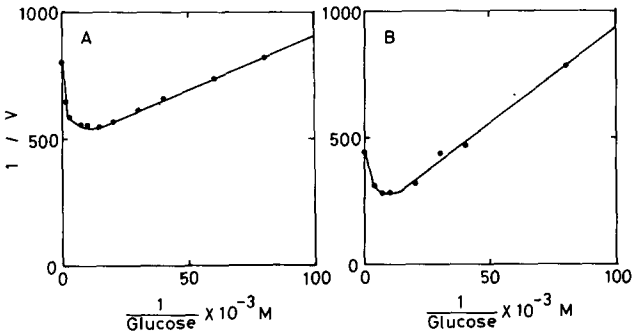

Fig. 5. Effect of glucose concentration on hexokinase D. A. The reaction velocity was measured with the system containing 2.5 mM ATP. B. 2.5 mM ADP.

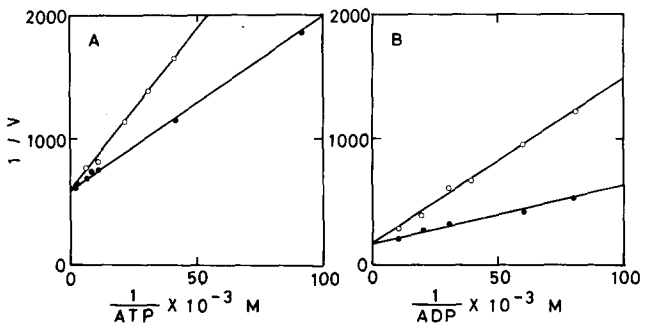

Fig. 6. Effect of concentration of ATP and ADP on hexokinase D and AMP inhibition. A. The reaction velocity was measured with the system containing 50 μM glucose, ATP of various concentrations, and 25 μM AMP (○———○) or without AMP (●———●). B. 50 μM glucose, ADP, and 12.5 μM AMP (○———○) or without AMP (●———●).

TABLE III

K_m AND K_i VALUES OF HEXOKINASE D OF RAINBOW-TROUT LIVER FOR SUBSTRATES AND INHIBITION AT 25°C AND pH 7.5

Compound	K_m (M)	K_i (M)
ADP	$1 \cdot 10^{-5}$	—
ATP	$2.7 \cdot 10^{-5}$	—
Glucose (with ADP)	$4.8 \cdot 10^{-5}$	—
Glucose (with ATP)	$8.8 \cdot 10^{-6}$	—
AMP (with ADP)	—	$1.1 \cdot 10^{-5}$
AMP (with ATP)	—	$1.4 \cdot 10^{-5}$

TABLE IV

EFFECT OF AMP ON HEXOKINASE D OF RAINBOW-TROUT LIVER

Activity was measured at pH 7.5 and 25°C in the presence of 2.5 mM ATP or ADP and 50 μM glucose

Concentration of AMP (M)	Hexokinase activity (%)	
	With ADP	With ATP
0	100	100
$2.5 \cdot 10^{-5}$	90	67
$5 \cdot 10^{-5}$	85	59
$1 \cdot 10^{-4}$	83	—
$2.5 \cdot 10^{-4}$	78	—
$5 \cdot 10^{-4}$	70	26
$1 \cdot 10^{-3}$	67	—
$2.5 \cdot 10^{-3}$	60	0

Although it is well known that hexokinase is inhibited by its reaction products, Glc-6-*P* and ADP [2,3], hexokinase D was found to be not inhibited by ADP. However, the enzyme was inhibited significantly by AMP. This inhibition was more pronounced with ATP as the phosphoryl donor than with ADP (Table IV). The other hexokinases (A, B and C) from fish liver are inhibited only slightly by AMP [1]. The type of inhibition of hexokinase D by AMP was competitive as shown in Fig. 6. The calculated K_i value for AMP in the ATP/glucose system was slightly higher than that in the ADP/glucose system (Table III).

Discussion

The results presented here show that the rainbow-trout liver contains two hexokinases, designated C and D from the elution pattern in DEAE-cellulose column chromatography. The properties of hexokinase D were similar to those of hexokinase III from rat liver [2] with respect to the K_m values for ATP and glucose and the substrate inhibition by glucose. On the other hand, hexokinase D from the rainbow-trout liver was found to have a wide specificity for nucleotides as phosphoryl donor. Furthermore, although ADP has been reported as an inhibitor of hexokinase [2,3], hexokinase D of the rainbow trout was not inhibited by ADP but capable of utilizing it as a phosphoryl donor. CTP, GTP, ITP and UDP were also phosphoryl donors for the enzyme, but the highest activity was observed when ADP was used. AMP, a reaction product from ADP, inhibited the enzyme. Limited utilization of ITP by brain hexokinase [8] and of some nucleoside triphosphates by yeast hexokinase [9] has been reported, but hexokinase capable of utilizing diphosphate is not known.

It is considered that the carnivorous fish such as rainbow trout and yellowtail are physiologically unable to utilize dietary carbohydrate of high concentration [10]. Phillips et al. [11] reported that the trout were normally diabetic since, after feeding of a sugar meal the blood glucose increase 110% with a curve which is similar to that of diabetic human being. The observation by Phillips et al. agrees with our findings that the trout liver contains no glucokinase (a hexokinase of high K_m for glucose), but two hexokinases C and D of low K_m for glucose, and the later enzyme is inhibited by glucose at high concentration. The natural diet of carnivorous fish, however, does not contain much carbohydrate, and hexokinase D in the liver might work for phosphorylation of glucose at a low level with ADP as well as ATP.

It is known that there are many factors to control the level of hexokinase activity, and each hexokinase has different kinetic properties and responds to environmental change in a different manner [12]. As we reported previously, the rainbow-trout liver contains no glucokinase but shows relatively high activity of glucose dehydrogenase [13]. The relationship between phosphorylation and oxidation of glucose in the liver of rainbow trout should be further investigated.

References

1 Nagayama, F. and Ohshima, H. (1974) Bull. Jpn. Soc. Sci. Fish. 40, 285—290
2 Grossbard, L. and Schimke, R.T. (1966) J. Biol. Chem. 241, 3546—3560

3 Fromm, H.J. and Zewe, V. (1962) J. Biol. Chem. 237, 1661—1667
4 Shatton, J.B., Morris, H.P. and Weinhouse, S. (1969) Cancer Res. 29, 1161—1172
5 Bandurski, R.S. and Axelrod, B. (1951) J. Biol. Chem. 193, 405—410
6 Ehira, S., Uchiyama, T. and Uda, F. (1974) in Analytical Methods in Fish Biochemistry and Food Chemistry (in Japanese) (Saito, T., Uchiyama, H., Umemoto, S. and Kawabata, T., eds.), pp. 17—31, Koseisha Koseikaku, Tokyo
7 Carter, C.E. (1950) J. Am. Chem. Soc. 72, 1466—1471
8 Joshi, M.D. and Jagannathan, V. (1966) Methods Enzymol. 9, 371—375
9 Purich, D.L., Fromm, H.J. and Rudolph, F.B. (1973) in Advances in Enzymology (Meister, A., ed.), Vol. 39, pp. 249—312, John Wiley and Sons, New York
10 Phillips, A.M., Jr. (1969) in Fish Physiology (Hoar, W.S. and Randall, D.J., eds.), Vol. 1, pp. 391—432, Academic Press, New York
11 Phillips, A.M., Jr., Tunison, A.V. and Brockway, D.R. (1948) N.Y. Conserv. Dept. Fish Res. Bull. 11, 44
12 Anderson, J.W., Herman, R.H., Tyrell, J.B. and Cohn, R.M. (1971) Am. J. Clin. Nutr. 24, 642—650
13 Nagayama, F., Ohshima, H. and Takeuchi, T. (1975) Bull. Jpn. Soc. Sci. Fish. 41, 1063—1067