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ISOLATION AND PROPERTIES OF PORCINE THYROID FUCOKINASE

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Summary

A 23 000-fold purification of porcine fucokinase (ATP:6-deoxy-L-galactose 1-phosphotransferase, EC 2.7.1.52) has been achieved using a combination of ion-exchange, hydrophobic ligand, affinity, hydroxyapatite and molecular sieve chromatography. The enzyme was determined to have a subunit molecular weight of 78 180 ± 4260 by sodium dodecyl sulfate chromatography and a tetrameric molecular weight of 309 200 ± 4100 in the active state as determined by molecular sieve chromatography. The enzyme exhibits a single pH optimum at a pH value of 6.5 and gives evidence of a high order of specificity for L-fucose and ATP. The enzyme requires a divalent metal ion and this need is best satisfied by Mg^{2+}. The activity of the enzyme is modified by a number of nucleotides. ADP is an enzyme inhibitor competitive with ATP. GDP-β-L-fucose is also an inhibitor and appears to compete with L-fucose. GDP-α-D-mannose stimulates the enzyme. A possible role for the actions of these nucleotide sugars is discussed.

Introduction

The enzyme fucokinase (ATP:6-deoxy-L-galactose 1-phosphotransferase, EC 2.7.1.52) has been found in a number of tissues and serves to utilize free L-fucose for glycoprotein synthesis. In thyroid tissue it undoubtedly is

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Abbreviations: Mops, 3-(*N*-morpholino)propanesulfonic acid; Taps, 3-[tris(hydroxymethyl)methyl]-aminopropanesulfonic acid; Caps, 3-(cyclohexylamino)propanesulfonic acid; TEMED, tetramethylethylenediamine.

instrumental in recycling L-fucose released by the physiological degradation of thyroglobulin. We have detected the enzyme in canine and porcine thyroid tissue and reported that the enzyme appears to be under metabolic control [1,2]. In this paper, we wish to report on the further purification of the porcine enzyme and its partial characterization.

Materials and Methods

Reagents

L-[1-^{3}H]Fucose and NaB^3H_4 were purchased from Amersham Corp. L-[1-^{14}C]Fucose was purchased from New England Nuclear. Sepharose 6B-100, horse spleen apoferritin (grade A), bovine pancreas α-chymotrypsinogen (type II), bovine hemoglobin (type II), bovine thyroglobulin (type I), sodium thioglycolate (grade V), (Mops, (Taps), (Caps) and $Na_2ATP \cdot 2H_2O$ were purchased from the Sigma Chemical Co. Cellex D, Bio Gel HT, ammonium persulfate, tetramethylethylenediamine (TEMED), *N,N'*-methylenebis-acrylamide, acrylamide, sodium dodecyl sulfate (SDS) and Coomassie brilliant blue R-250 were purchased from Bio Rad Laboratories. Seakem agarose was purchased from MCI Biomedical. Agarose-ϵ-aminocaproyl-β-fucosamine was purchased from Miles Yeda. SB-2 anion-exchange resin-loaded paper was purchased from Reeve Angel Corporation. Beef liver catalase was purchased from Worthington Biochemical Corporation. L-Galactose and D-[1-^{14}C]galactose were gifts from Drs. George Barber and Patricia Hebda, Ohio State University. GDP-β-L-fucose was a gift from Drs. R. Barker and P. Rosevear of the Dept. of Biochemistry at Michigan State University. The fresh porcine thyroid glands were obtained through the courtesy of the Ohio Packing Co., Columbus, OH.

pH measurement

The pH at 37°C was measured with the meter calibrated at 37°C and the electrode kept in 37°C water between measurements. The pH at 23°C was measured in an analogous manner. The buffers used in the pH optimum studies were sodium acetate at a pH of 4—6, imidazole at a pH of 6—6.5, Mops at a pH of 6—7.5, Taps at a pH of 7—8.5, and Caps at a pH of 8.5—10. All pH values in the optimum study were measured at the temperature used in the fucokinase assay, i.e. 37°C.

Fucokinase assay

The assay procedure was essentially that of Richards and Serif [1] with minor modification. The final concentrations of assay components were: $MgSO_4$, 6 mM; ATP, 5 mM KF, 8.3 mM; glycerole, 1.0 M; Mops, 13.3 mM at the appropriate pH; β-mercaptoethanol, 3.3 mM; L-fucose, 0.6 mM (containing 22 mCi of L-[1-^{3}H]fucose/mmol of L-fucose). A range of MgATP concentrations from 0.5 M to 5 M was used with the stimulatory GDPsugar studies. Protein concentrations were determined using the fluorescamine procedure. [3]. One unit of fucokinase is the amount of enzyme needed to form 1 nmol of fucose 1-phosphate/min at a pH of 7.44 and 37°C. The product of enzymic action was shown to be β-fucose 1-phosphate by chromatography procedures already described [1].

Molecular weight determination

The monomer molecular weight was determined using acrylamide gels in a continuous phosphate system containing SDS (7% acrylamide, 0.19% bis-acrylamide, 0.1% SDS, 0.15% TEMED, 0.037% ammonium persulfate and 100 mM sodium phosphate, pH 7.2). The running buffer contained 0.1 M sodium phosphate and 2.0% SDS at a pH of 7.2. Samples were prepared for electrophoresis by mixing 1 : 1 with a denaturing buffer containing 0.02 M sodium phosphate (pH 7.2), 1% SDS, 20% glycerol and 100 mM dithiothreitol and heating at 100° C for 2.5 min. Electrophoresis was carried out at 3 mA/gel until the sample entered the gel, then 6 mA/gel for 5 h at 23° C. After electrophoresis, gels were stained for protein by the procedure of Fairbanks et al. [4]. Cross-linked ovalbumin molecular weight standards were prepared by the method of Payne [5]. Labeled samples for monomer molecular weight determination were prepared by a modification of the method of Rice and Means [6]. The sample to be labeled, containing approximately 1 μg of protein in 10 μl, was added to 20 μl of 0.1 M borate buffer (pH 10.4). A 1.8 μl volume of a formaldehyde/water solution (1 : 71) was added to the buffered sample which was mixed and placed on ice. After 2 min 0.6 mCi (5 μl) of a NaB^3H_4 solution (7 Ci/mmol in 0.02 M NaOH) was added with shaking. The reduction was permitted to proceed for 10 min. The reaction mixture was then dialyzed against four 500-ml vols. of distilled water for 45 min with each new change of water. The dialyzed sample was then mixed with denaturing buffer and electrophoresed as described above. The gel containing labeled monomer fucokinase was stained for protein to visualize the bands and then sliced into 2-mm sections. Each gel slice was placed in a glass scintillation vial which contained 0.9 ml of a water/Protosol solution (New England Nuclear Corp., Boston) 1 : 9. The vials were capped and kept at 37° C overnight such that the gel slice was submerged in the Protosol solution. After cooling the samples, 10 ml of Aquasol (New England Nuclear Corp., Boston, MA) were added and the samples counted when chemiluminescence had subsided.

The molecular weight of the oligomer was determined by molecular sieve chromatography. A Sepharose 6B-100 column was prepared (307 ml bed volume, inner diameter 2.5 cm, height 64 cm), topped by 1 cm layer of BioGel P-2. The column was calibrated using horse spleen apoferritin, ovalbumin, bovine pancreas α-chymotrypsinogen, bovine hemoglobin, bovine thyroglobulin, bovine serum albumin and bovine liver catalase. Samples were applied in a 10 ml volume and 2-ml fractions were collected.

Purification of fucokinase

The following procedure represents a typical example from three separate isolations of fucokinase from porcine thyroid tissue. All operations were conducted at 4° C.

Preliminary preparation of tissue extracts. Fresh porcine thyroids (approx. 47 glands, 300 g) were excised from recently killed pigs and carried to the laboratory on ice. The glands were cleaned of all visible pieces of fat or membranous material, sectioned into large pieces and passed through a cooled meat grinder. The finely ground tissue was collected in a Polytron-equipped Waring blender jar containing 150 ml of 0.02 M Mops, 0.005 mM β-mercaptoethanol

and 1.0 M glycerol at a pH of 7.4 (buffer A). When it appeared that the majority of the tissue was minced the grinder was dismantled and pieces of the tissue which adhered to the inside of the grinder were removed and added to the tissue in the blender jar. Homogenization was accomplished by three 20-s periods at high speed with interceding 20-s periods of cooling. The tissue slurry was passed through four layers of cheesecloth and the residue was squeezed by hand to remove as much liquid as possible. The filtrate (375 ml) was centrifuged at 12 000 × *g* (3°C, 30 min). The 12 000 × *g* supernatant solution was filtered through glass wool and the pellet discarded. The supernatant solution was subsequently centrifuged at 227 430 × *g* (3°C, 60 min). The solution derived from this treatment was filtered through glass wool prior to column chromatography.

DEAE-cellulose chromatography. The 227 430 × *g* supernatant solution was passed into a 200 ml DEAE-cellulose column (5.5 cm × 8 cm). After the sample had entered the column an elution regimen was followed, which sequentially included 1050 ml of buffer A, 400 ml of a 0—0.15 M KCl linear gradient in buffer A, 1370 ml of 0.15 M KCl in buffer A, a 500 ml 0.15—0.60 M KCl linear gradient in buffer A, and 745 ml of 0.6 M KCl in buffer A. Fractions of 20 ml were collected with the majority of the fucokinase activity eluting during the 0.15—0.6 M KCl gradient (Fig. 1). The fractions highest in fucokinase activity were combined (445 ml) and 3.8 g of solid KCl added/100 ml of solution. The KCl was added in small portions with stirring to prevent extended exposure of the enzyme to undissolved KCl.

Octyl Sepharose CL-4B chromatography. The 0.66 M KCl preparations from the previous step was passed through an Octyl Sepharose column with a bed volume of 120 ml (5 cm × 10 cm). After the sample had entered the column an elution regimen was followed which consisted of 270 ml of 1 M KCl in buffer A and then 400 ml of buffer A. This elution procedure must take place as

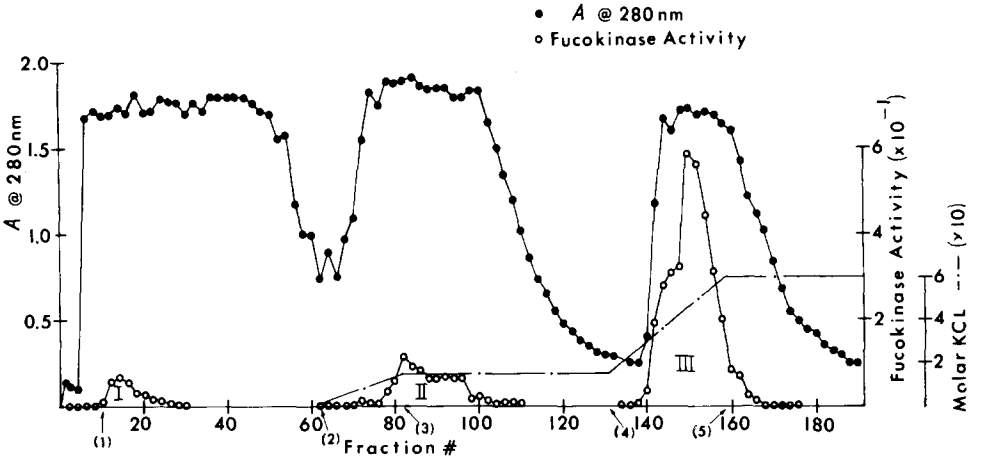


Fig. 1. Elution of a porcine thyroid 47 500 rev./min supernatant solution from a DEAE-cellulose column. Fucokinase activity is expressed as nmol of fucose 1-phosphate formed/60 min per ml assay mixture. The peak I area represents 6% peak II area 18% and the peak III area 76% of the combined peak areas. (1) Buffer A was; (2) 0—0.15 M KCl in buffer A gradient; (3) 0.15 M KCl in buffer A wash; (4) 0.15—0.60 M KCl in buffer A gradient, and (5) 0.60 M KCl in buffer A wash.

rapidly as possible to insure the maximum recovery of fucokinase activity. The fucokinase activity was eluted in the buffer wash (Fig. 2). The fractions (20 ml) highest in fucokinase activity were combined (135 ml). These pooled fractions were then dialyzed against buffer A until KCl was no longer detected in the dialysis buffer (four changes of 1 l of buffer A with 1 h between changes).

Agarose-ϵ-aminocaproyl-β-fucosamine chromatography. The dialysate from the previous step (135 ml) was passed through a column of agarose-ϵ-aminocaproyl-β-fucosamine (1.5 cm × 7.3 cm) with a 10 ml bed volume. After the sample had entered the column an elution regimen was followed which consisted of sequential elution by 67 ml of buffer A, 117 ml of 0.08 M KCl in buffer A, and 64 ml of 0.45 M KCl in buffer A. 8-ml fractions were collected except for 4-ml fractions during the 0.45 M KCl wash. The 0.08 M KCl allowed some fucokinase to leak off the column. Consequently this was continued only until the large peak of protein eluted (Fig. 3) and the absorbance at 280 nm was a constant near-zero figure. The fucokinase eluted with the 0.45 M KCl in buffer A and the fractions highest in fucokinase activity were combined and dialyzed against buffer A until no KCl was detected in the dialysis buffer (three changes of 1 l of buffer A with 2 h between changes).

Hydroxyapatite column chromatography. The dialyzed material from the previous step (24 ml) was concentrated to approximately 1 ml using a 10 ml Amicon filtration cell with a PM-10 Diaflo ultrafilter under nitrogen pressure (Amicon Corp., Lexington, MA). This concentrated material was passed over an hydroxyapatite column (1 cm × 2.3 cm) with a 1.5 ml bed volume and eluted sequentially with 8 ml of buffer A and 20 ml of a linear gradient of 0—0.10 M phosphate in buffer A. Fractions of 1 ml were collected. Fucokinase eluted from the column at a 25 mM phosphate concentration (Fig. 4) and the fractions highest in fucokinase activity were combined and dialyzed against buffer A (two changes of 250 ml with 2 h between changes).

Sepharose 6B-100 chromatography. The dialyzed preparation from the previous step was placed on a Sepharose 6B-100 column (2.5 cm × 58 cm) with

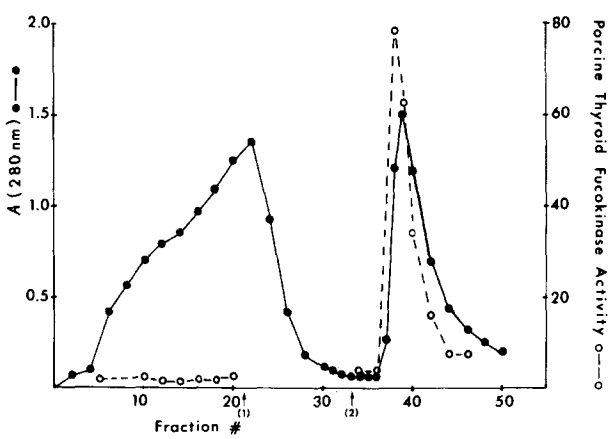


Fig. 2. Elution of a porcine thyroid DEAE dialysate preparation from an Octyl Sepharose column. Fucokinase activity is in units of nmol of fucose 1-phosphate formed/60 min per ml assay mixture. (1) 1 M KCl in buffer A wash; (2) buffer A wash. No elution of fucokinase occurs with the 1 M KCl buffer wash.

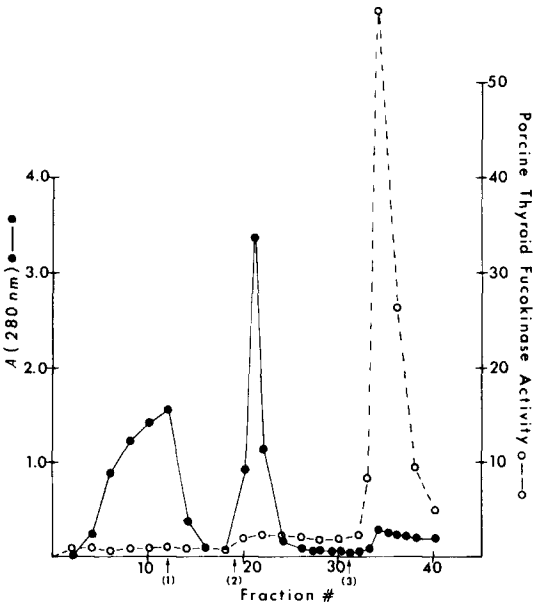


Fig. 3. Elution of a porcine thyroid Octyl Sepharose dialysate from the affinity column. Fucokinase activity is in units of nmol fucose 1-phosphate formed/60 min per ml assay mixture. Fraction volumes are 8 ml up to fraction 31 and 4 ml thereafter. (1) Buffer A wash; (2) 0.08 M KCl in buffer A wash, and (3) 0.45 M KCl in buffer A wash.

a 310 ml bed volume. The column had previously been equilibrated with buffer A. A 1 cm layer of the BioGel P-2 topped the column. The column was eluted with buffer A and 2-ml fractions were collected. The fucokinase was eluted from the column in a symmetrical peak (Fig. 5). The fractions highest in fucokinase activity were combined and concentrated by ultrafiltration.

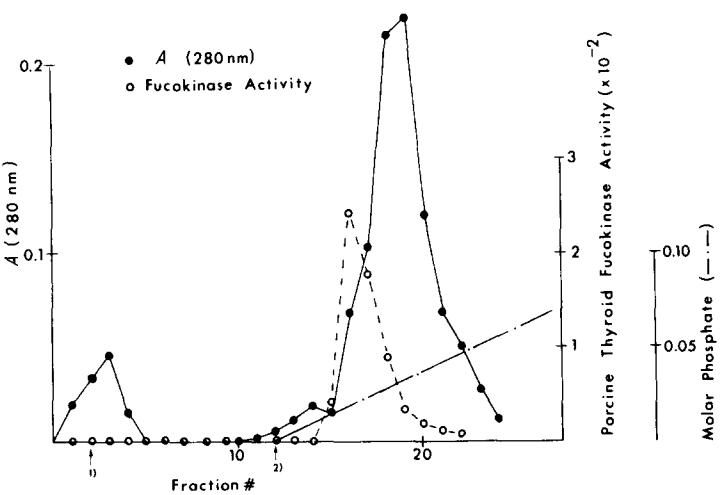


Fig. 4. Elution of a porcine thyroid affinity column dialysate from a hydroxyapatite column. Fucokinase activity is expressed as nmol of fucose 1-phosphate formed/60 min per ml assay mixture. (1) Buffer A wash, and (2) 0—0.10 M phosphate in buffer A gradient.

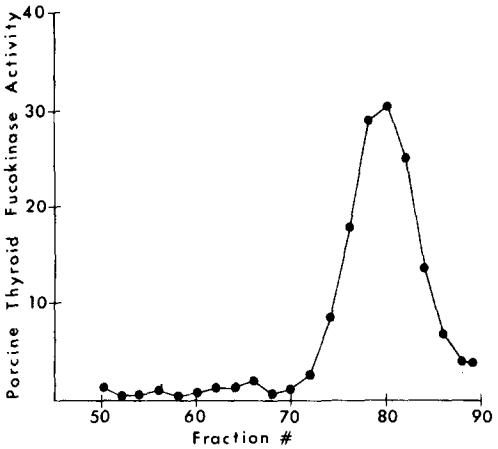


Fig. 5. Elution of a porcine thyroid hydroxyapatite dialysate from a Sepharose 6B column. Fucokinase activity is in units of nmol of fucose 1-phosphate formed/180 min per ml assay mixture. Fractions 70—90 exhibit no absorbance at 280 nm prior to concentration.

Results

Enzyme purification

A typical yield and purification sequence for fucokinase prepared by the regimen described in Materials and Methods is presented in Table I. The overall purification of the enzyme is 23 000-fold relative to its specific activity in the crude extract. Samples of fucokinase derived from step 6 (Table I) of the purification process were electrophoresed in 2% acrylamide/0.5% agarose gels. Pre-electrophoresis was carried out at 4 mA/gel for 2 h at 3°C while electrophoresis was conducted at 2 mA/gel for 1 h and 4 mA/gel for 10 h at 3°C. These gels were split in half longitudinally and one-half stained for protein and

TABLE I

PURIFICATION TABLE FOR PORCINE THYROID FUCOKINASE

Purification step		Volume (ml)	Total protein (mg)	Percent yield	Specific activity (units/mg)	Total activity	Purification (-fold)
1	12 100 × *g* supernatant	235	12 200	100	0.026	318	—
2	227 430 × *g* supernatant	220	10 428	92	0.028	292	1
3	DEAE-cellulose dialysate [a]	750	787	118	0.48	376	18
4	Octyl Sepharose dialysate [b]	85.5	74	20	0.86	64	33
5	Fucose affinity column dialysate [c]	23.2	3.7	5	4.63	17	178
6	Hydroxyapatite dialysate [d]	3.2	0.19	2	32.02	6	1 231
7	Sepharose 6B concentrate	—	0.01	2	600	6	23 078

[a] Fucokinase eluted with a 0.15—0.60 M KCl in buffer A linear gradient.
[b] Fucokinase eluted with a buffer A wash.
[c] Fucokinase eluted with a 0.45 M KCl in buffer A wash.
[d] Fucokinase eluted with a 0—0.10 M phosphate in buffer A linear gradient.

the remaining half examined for enzyme activity [1]. The major peak of protein coelectrophoresed with the enzyme activity, although a prominent second peak was also observed. Electrophoresis of the intact enzyme was attempted after the final step in enzyme purification (step 7). However, the protein concentration was so low that protein staining to detect the protein band was unsuccessful. Since SDS electrophoresis generally provides sharper protein bands, it was decided that protein from step 7 (Table I) would be examined by means of SDS electrophoresis (see Materials and Methods). Prior to this examination, however, the protein was labeled with tritium by reductive methylation (see Materials and Methods). After labeling and SDS electrophoresis the gel was stained with Coomassie blue R-250 [4]. This stain produced a single faint blue band. Since the intensity of the band was weak, it was difficult to assess whether other minor bands might be hidden. Consequently the gel was sliced into 1 mm sections and each section counted by liquid scintillation techniques. The radioactive profile obtained is presented in Fig. 6. A single major peak is observed at an R_F of 0.34. A minor shoulder peak observed an R_F of 0.43 may represent a small impurity in the preparation or a degradation product of the major peak due to the SDS or reductive methylation treatments.

Step 5—7 fucokinase preparations (Table I) were quite stable to storage at —20° C by freezing in a solid CO_2/acetone bath in the presence of mercaptoethanol and glycerol. The enzyme was particularly unstable in the presence of KCl and similar salts such that maintenance of the enzyme in saline solutions for long periods at 4° C or freezing in the presence of KCl irreversibly denatured it. As a consequence particular care was necessary in the Octyl Sepharose and affinity column steps to minimize the period of exposure to KCl and to remove the salt prior to freezing. Although the Octyl Sepharose column step is a poor one due to inactivation of enzyme by this exposure to KCl, it did remove several prominent extraneous bands of protein (detected electrophoretically) which were not removed by other procedures. Consequently this step was retained.

Fucose dehydrogenase activity was absent from our preparations at the fucose affinity column step. Yurchenco and Atkinson report that fucose

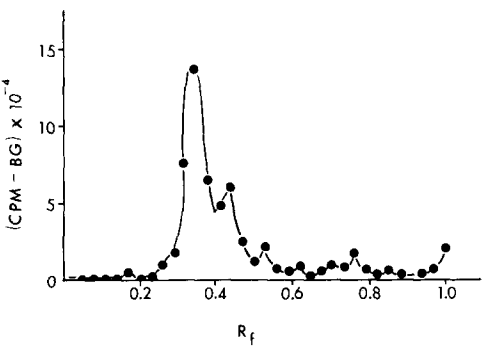


Fig. 6. Radioactivity profile of an SDS electrophoresis gel of the Sepharose 6B fucokinase preparation prelabeled with tritium by reductive methylation.

dehydrogenase was removed at a DEAE step in their preparation of porcine liver fucokinase [12]. It is possible by analogy that this enzyme, responsible for the initial step in catabolic breakdown of fucose, may have been removed at our earlier DEAE step as well.

Monomer and oligomer molecular weight

A standard molecular weight curve was developed for the SDS electrophoresis gels as described in Methods. Fitting a 'least means square' line to this curve gave the expression:

$$\text{Log}_{10} M_r = -1.183\ (R_F) + 5.319$$

An average R_F of 0.36 ± 0.02 was obtained for the monomer. From these data a monomer molecular weight of 78 180 ± 4260 was calculated. A determination of the oligomer molecular weight by molecular sieve chromatography gave a value of 309 200 ± 4100. On the basis of these data the oligomer appears to be a tetrameric aggregate of a single monomeric unit.

pH optimum

A pH optimum curve was established for the enzyme as described in Materials and Methods. This curve contains a single optimum peak with a narrow plateau between the pH values 6.3 and 6.9. The decrease in optimum is abrupt below 6.3 with irreversible denaturation of the enzyme occurring below a pH value of 6.0. Thus although the assay optimum is at pH 6.6, a pH of 7.4 was used in enzyme isolation buffers to minimize denaturation.

Substrate specificity

A step 6 enzyme preparation (Table I) was used to examine the substrate specificity of porcine focukinase. This preparation did not accept D-[^{14}C]-mannose, D-[^{14}C]glucose, D-[^{14}C]galactose, D-2-deoxy-[^{14}C]glucose, D-[^{14}C]-xylose or D-[^{3}H]ribose as substrates since no measurable phosphorylation of these sugars was observed when they were substituted for labeled L-fucose in the normal assay.

Other sugars which might serve as substrates because of their structural similarity to L-fucose were unfortunately not available in labeled forms. Thus D-arabinose, in the pyranose form, resembles the ring system and carbons 1—5 of L-fucose while L-galactose is the 6-hydroxy form of L-fucose. In the absence of these labeled derivatives an alternative method was employed to test their effectiveness as substrates. If these unlabeled sugars are substrates for the kinase they will compete with labeled L-fucose for the active site. In that circumstance the addition of the unlabeled sugar to a normal labeled L-fucose assay should produce inhibition of labeled L-fucose conversion to β-L-fucose 1-phosphate. These competition studies, although useful in investigating the specificity of the enzyme, would not reveal the presence or absence of kinase contaminants. Table II presents the results of such a study. The only sugar which induces an inhibition of labeled L-fucose is unlabeled L-fucose. Thus it appears that porcine fucokinase is highly specific for L-fucose.

Reciprocal velocity versus reciprocal fucose concentration curves were plotted for the step 6 enzyme preparations (Table I) using eh computer

TABLE II

SUBSTRATE SPECIFICITY USING UNLABELED SUBSTRATES

The unlabeled sugar was present at 800 μM concentration and the fucose concentration in each assay vial was 600 μM.

Unlabeled sugar added	% of cpm
None	100
L-Fucose	37
D-Arabinose	103
L-Galactose	111
L-Rhamnose	96

program of Wilkinson [7]. Extrapolation of these curves to the 1/[fucose] axis gives an apparent K_m value of 30 μM for fucose.

The effectiveness of nucleoside triphosphates as phosphate donors was also examined with porcine fucokinase. CTP, UTP, GTP and TTP were determined to be completely ineffective within the limits of the enzyme assay (less than 1% of the effectiveness of ATP). Addition of these compounds to a normal assay containing ATP as the phosphate donor (Table III) caused inhibitory effects in some cases, suggesting non-productive competition for the ATP site.

Reciprocal velocity versus reciprocal MgATP concentration curves were plotted for the step 6 (Table I) enzyme preparations using ATP:Mg^{2+} ratios of 1 : 2 [1]. Extrapolation of these curves to the 1/[MgATP] axis gave an apparent K_m value of 1.0 mM for the MgATP complex.

Influence of various metabolites

The effect of a series of monophospho- and diphosphonucleosides on porcine fucokinase is presented in Table IV. Only ADP is seen to be a potent inhibitor of the enzyme. The nature of this product inhibition was examined kinetically. An examination of the double-reciprocal plot of Fig. 7 clearly establishes that the inhibition is a competitive one between ADP and ATP.

Since GDP sugars uniquely influence fucokinase, as reported in a preliminary communication [2], we undertook to examine the nature of their effects more

TABLE III

INFLUENCE OF ADDED NTP ON PORCINE THYROID FUCOKINASE ACTIVITY IN THE PRESENCE OF ATP

Concentration given is in the assay mixture. Mg^{2+}:NTP ratio is 10:6.

NTP added	Concentration (mM)	Percentage of activity
None	—	100
ATP	4.7	104
CTP	4.9	70
TTP	4.8	55
UTP	4.9	116
GTP [a]	0.10	41

[a] Electrophoretically purified.

TABLE IV

INFLUENCE OF ADDED NDP OR NMP ON PORCINE THYROID FUCOKINASE ACTIVITY IN THE PRESENCE OF ATP

Concentration given is in the assay mixture. Mg^{2+}:NXP ratio was 1:1.

Nucleotide added	Concentration (mM)	Percentage of activity
None	—	100
ADP	4.9	4
AMP	5.0	86
CDP	4.9	84
CMP	4.9	103
GDP [a]	0.10	91
GMP	5.0	136
TDP	5.0	100
UDP	4.9	98
UMP	5.0	105
Cyclic AMP	4.9	97
Cyclic GMP	5.0	114
Cyclic GMP	$1.35 \cdot 10^{-5}$	102
Cyclic GMP	$1.35 \cdot 10^{-7}$	108

[a] Electrophoretically purified.

closely using GDP-α-D-mannose and GDP-β-L-fucose as examples of the stimulatory and inhibitory nucleotide sugars, respectively. The Lineweaver-Burk plot of Fig. 8 shows the stimulatory effect of GDP-α-D-mannose on fucokinase. Only slight differences are observed in the K_m of fucokinase in the presence of GDP-α-D-mannose (37 μM) as opposed to its absence (30 μM). On the other hand, the enhancement of V is greater than two-fold. Fig. 9 contains a similar plot of the effect of GDP-β-L-fucose on fucokinase. Here the nucleotide sugar is observed to strongly inhibit the fucokinase and the inhibition is seen to be of a competitive nature.

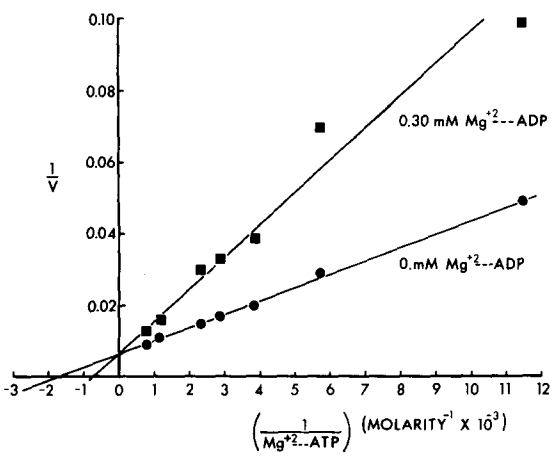
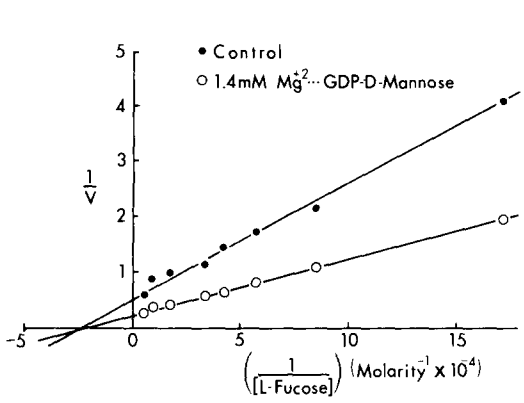


Fig. 7. Lineweaver-Burk plots showing the effect of ADP as a competitive inhibitor of ATP in the fucokinase-catalyzed reaction.



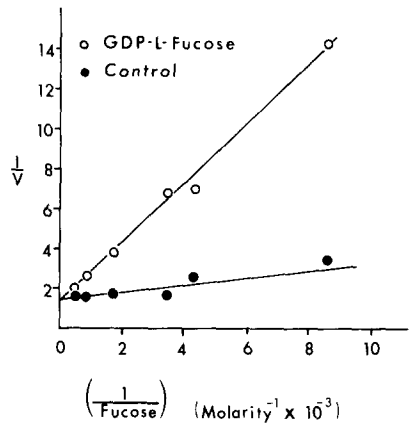


Fig. 8. Lineweaver-Burk plots showing the stimulatory effects of GDP-α-D-mannose on the fucokinase-catalyzed reaction.

Fig. 9. Lineweaver-Burk plots showing the competitive inhibition by GDP-β-L-fucose of fucose 1-phosphate formation by the fucokinase-catalyzed reaction.

Discussion

Previous work with the fucokinase from canine thyroid [1] suggested that the enzyme was much more specific with respect to the sugar moiety than had been previously reported for the porcine liver enzyme [8,12]. However, even the canine thyroid enzyme at its state of purity did phosphorylate both D-mannose and D-glucose to an appreciable extent although it was inferred that this phosphorylation was in the 6-position of the sugar rather than the 1-position and represented impurities in the enzyme of the 'hexokinase' type. The porcine thyroid enzyme at step 6 in the purification scheme and above (Table I) shows no tendency to phosphorylate any sugar tested other than L-fucose and thus appears to possess a high order of specificity with respect to L-fucose. Why such a higher order of specificity has been developed is not readily apparent. Does the enzyme normally encounter alternate sugars with similar structures which if phosphorylated could cause metabolic confusion? Certainly few mammalian enzymes phosphorylating six-carbon sugars on the acetal hydroxyl group have been detected to date [9].

Significant species differences exist between the canine and porcine thyroid fucokinases. The molecular weight determined for the canine enzyme was 494 000 [1] while that for the porcine enzyme appears smaller (309 200). Whether this dissimilarity represents differences in aggregation of subunits or considerably different subunit sizes is not presently determinable since the canine enzyme is not available in a sufficiently pure state to establish its subunit size. The pH profile for the two enzymes is also unlike. Both enzymes exhibit a pH optimum near 6.6 but only the canine enzyme exhibits an optimum at pH 8.25.

Both porcine and canine fucokinase appear susceptible to modulation by a variety of metabolites. The most interesting of these appear to be the GDP sugars. With the porcine enzyme, the effect of GDP-β-L-fucose would appear to

result from its interference with the L-fucose binding site since competitive kinetics are observed with that substrate. The mechanism for the action of GDP-α-D-mannose is much less obvious. Since the apparent K_m for the enzyme is not greatly affected by the nucleotide sugar it appears that the major effect of GDP-α-D-mannose is to cause an increase in the apparent V for the enzyme. The Hill plots for the data of Fig. 8 give slopes of 0.77 for the untreated enzyme and 1.0 for the GDP-α-D-mannose activated system. These data imply a moderate negative cooperativity with the unstimulated fucokinase which is relieved in the presence of GDP-α-D-mannose. The modest nature of this effect, however, is unlikely to account for the activating effects of the nucleotide sugar. Thus at present the allosteric mechanism for the GDP-α-D-mannose stimulation is unknown.

The rationale for the observed stimulatory action of GDP-α-D-mannose on fucokinase in vitro is also not completely clear, although the possibility of a regulatory function in vivo exists. β-L-Fucose 1-phosphate, the product of fucokinase action, can undoubtedly be converted to GDP-β-L-fucose in the thyroid gland [10]. This nucleotide sugar and GDP-α-D-mannose are required for the synthesis of some of the same polysaccharide units in various glycoproteins, e.g. polysaccharide B of thyroglobulin [11]. In view of this fact, an interpretation of the data might be that the stimulation of fucokinase by GDP-α-D-mannose may lead to enhanced synthesis of GDP-β-L-fucose and may represent the physiological interaction of two pathways contributing to the synthesis of one product, e.g. thyroglobulin polysaccharide B. This interpretation seems to ignore the fact that GDP-α-D-mannose may be required in the synthesis of other glycoproteins that do not contain L-fucose where the stimulatory action of GDP-α-D-mannose on fucokinase would appear to be irrelevant. However, it could be hypothesized that when a GDP-β-L-fucose concentration is generated in excess of that required for glycoprotein formation, GDP-β-L-fucose would partially limit further synthesis of itself, at least by the salvage pathway, by feedback inhibition of fucokinase, countering the action of GDP-α-D-mannose. Further study is required to establish the authenticity of such a system and, indeed, to verify the importance of the salvage pathway in the overall metabolism of L-fucose.

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