Isozymic Composition and Regulatory Properties of Phosphofructokinase from Well-Differentiated and Anaplastic Medullary Thyroid Carcinomas of the Rat

Ralph Oskam, Gert Rijksen, Gerard E. J. Staal, and Shobhana Vora

Division of Medical Enzymology, Department of Haematology, Academic Hospital, Catharijnesingel 101, 3500 CG Utrecht, The Netherlands [R. O., G. R., G. E. J. S.], and Department of Basic and Clinical Research, Scripps Clinic and Research Foundation, La Jolla, California 92037 [S. V.]

ABSTRACT

Acceleration of glycolysis is, in general, a characteristic of neoplasia. Previous studies have shown that this increase in glycolysis is achieved by quantitative increases in the activities of the key regulatory enzymes, hexokinase, phosphofructokinase (PFK) and/or pyruvate kinase, which are often accompanied by isozymic alterations that facilitate glycolysis. In this study, we investigated the alterations in the activity, isozymic profile, and kinetic-regulatory properties of PFK from the medullary thyroid carcinomas of the rat, which represent a model for the neuroectodermally derived tumors in humans.

Contrary to the expected, we found that undifferentiated tumors showed a decrease in the enzyme activity as compared to the highly differentiated tumors. This decrease in PFK activity was accompanied by an increase in the expression of the liver-type isozyme of PFK. The enzymes from the 2 tumor types showed no significant differences in their affinity and cooperativity toward the substrates, fructose-6-phosphate and adenosine triphosphate (ATP). However, the tumor PFKs showed major differences with respect to their behavior toward the allosteric regulators of the enzymes, ATP, citrate, and fructose-2,6-diphosphate; the latter is a recently discovered activator of the enzyme. The enzyme from the undifferentiated tumor was less sensitive to citrate inhibition, which was more readily reversed by cyclic adenosine 3':5'-monophosphate. In addition, it was less sensitive to ATP inhibition at low fructose-6-phosphate concentrations. More importantly, the enzyme from the undifferentiated tumors was more sensitive to the activation by fructose 2,6-diphosphate especially when inhibited by citrate and ATP. The altered regulatory properties of the enzyme from the undifferentiated tumors most probably reflect its altered isozymic composition, i.e., increase in the liver-type isozyme. The preferential expression of the liver-type isozyme by undifferentiated and rapidly replicating cancer cells may be explained in terms of the unique regulatory properties of this isozyme. Although the concentrations of fructose 2,6-diphosphate were comparable in these 2 tumor types, the higher sensitivity of the liver-type PFK to activation by this compound may permit accelerated glycolytic flux observed in undifferentiated tumors, despite a decrease in total PFK activity.

INTRODUCTION

PFK (ATP: d-fructose 6-P, 1-phosphotransferase; EC 2.7.1.11), one of the key rate-limiting enzymes of glycolysis, catalyzes the phosphorylation of Fru-6-P to Fru-1,6-P2 in the presence of ATP and Mg2+. The regulation of PFK activity is complex and is controlled by a number of metabolites, hormones, and nutritional states such that the rates of glycolysis are altered in accord with the cellular need for energy and/or glycolytic intermediates (2, 38). Thus, PFK plays a critical role in the energy metabolism of organs largely or entirely dependent upon glycolysis, i.e., mature red cells, exercising muscle, brain (under most conditions), ischemic heart, and neoplastic cells.

Mammalian PFK is a tetrameric protein consisting of 4 identical or nonidentical subunits. Extensive and elegant studies of PFK from humans and the rabbit have shown that the enzyme is under the control of 3 loci, which code for M, L, and P subunits. These subunits are differentially expressed by different organs and undergo random tetramerization to produce various homo- and heterotetrameric isozymes. These isozymes are distinctly identifiable from one another, based upon their distinct physicochemical, immunochemical, and kinetic-regulatory properties (5, 14, 21, 36, 37, 43, 46). In contrast, until recently, it was unclear whether the rat PFK is also under the control of 2 or 3 structural loci; the existence of the third brain-type subunit was not conclusively established. Using the techniques of ion-exchange chromatography and subunit-specific antisera, we have recently demonstrated that the rat PFK is also under the control of 3 loci, as is the case with humans.

The carbohydrate metabolism of cancer cells is characterized by the predominance of aerobic glycolysis over gluconeogenesis, presumably to meet increased energy requirements and to facilitate the production of ribose-5-phosphate (via hexose monophosphate shunt) for increased DNA synthesis (49, 50). In experimental rat hepatomas of varying degrees of malignancy, i.e., differentiation and growth rate, etc., 2 of the key glycolytic enzymes, i.e., PK and HK, show not only quantitative increases but also isozymic shifts that appear to facilitate glycolysis. In contrast, although PFK activity also increases, the highly regulated PFK isozyme, i.e., liver type, not only persists but actually...
increased significantly (44, 45).

In order to understand the physiological role(s) and significance of the altered gene expression of PFK in neoplasia, in this study, we investigated the spontaneously developed medulIary thyroid carcinomas of the rat which afford a model system for the human (neuro-) ectodermally derived neoplasias. We have studied the activity, isozymic profile, and kinetic-regulatory properties of the enzyme from the tumors of differing degrees of differentiation with special reference to the recently discovered regulator of glycolysis, Fru-2,6-P2 (11, 26, 40). Our results indicate that, as reported previously, the highly regulated liver-type isozyme increases in its expression with increasing degree of dedifferentiation of the tumor but, unlike previously observed increases in PFK activity, the enzyme activity actually decreases in this tumor type. Despite a diminution of PFK, the observed high glycolytic rate(s) of the tumor (28) is probably maintained due to the increased sensitivity to Fru-2,6-P2 activation of the liver-type isozyme, since the intratumoral levels of the compound are comparable in differentiated and undifferentiated tumors. These results are suggestive of a novel role for Fru-2,6-P2 in the regulation of glycolysis in cancer cells.

**MATERIALS AND METHODS**

Adenine nucleotides, NADH, Fru-6-P, Fru-1,6-P2, Fru-2,6-P2 and DTT were purchased from Sigma Chemical Co. (St. Louis, MO). Fructose diphosphate aldolase, α-glycerol-3-phosphate dehydrogenase, and triosephosphate isomerase were from Boehringer Mannheim (Mannheim, Federal Republic of Germany). DEAE-Sephadex A-25 was from Pharmacia Fine Chemicals (Piscataway, NJ). Nonidet P-40 was purchased from Particle Data (Chicago, IL), and Staphylococci-bearing protein A (IgG-Sorb) were from The Enzyme Center (Boston, MA). All other chemicals were of reagent grade.

**Assays of PFK Activity.** For the studies defining PFK activities of the normal tissues and tumors of the rat, PFK was assayed kinetically in a final volume of 1 ml at 37°C in a Beckman 35 spectrophotometer by measuring the decrease in absorption at 340 nm in the enzyme coupled assay using 1.4 units aldolase, 1.5 units α-glycerol-3-phosphate dehydrogenase, 4.5 units triosephosphate isomerase, and 0.2 mM NADH. Maximal velocities were measured in 100 mM Tris-HCl buffer (pH 8.0), containing 10 mM KCl, 5 mM MgCl2, 5 mM (NH4)2SO4, 1 mM EDTA, 5 mM DTT, 2 mM Fru-6-P. After incubation with sample, the reaction was started with 0.5 mM MgATP. One unit of PFK activity is defined as that amount of enzyme that converts 1 μmol Fru-6-P into Fru-1,6-P2 in 1 min in the above system. For the chromatographic and immunological studies, PFK activity was assayed at 26°C as described previously (46). Activities of HK and PK were determined enzymatically as described before (28). G6PD activity was assayed kinetically in 100 mM Tris-HCl buffer (pH 8.0) containing 10 mM MgCl2, 0.2 mM NADP, and 0.6 mM glucose 6-phosphate.

**Kinetic Studies of PFKs.** Kinetic studies were performed at 37°C in 50 mM glycylglycine:KOH buffer (pH 7.35) containing 50 mM KCl, 1.5 mM MgCl2 (unless otherwise specified), 0.5 mM (NH4)2SO4, 0.5 mM EDTA, 5 mM DTT. BSA (1 mg/ml); aldolase (1.4 units/ml), glycerol-3-phosphate dehydrogenase (1.5 units/ml), triosephosphate isomerase (4.5 units/ml), 0.2 mM NADH, and the indicated concentrations of MgATP, Fru-6-P, and other effectors of PFK. Reactions were started as indicated with either Fru-6-P or MgATP or the enzyme.

Excess ammonium sulfate was removed from the partially purified PFK preparations and from the auxiliary enzymes by extensive dialysis against chilled 20 mM Tris-phosphate buffer (pH 7.5), containing 10 mM (NH4)2SO4, 10 mM potassium fluoride, 5 mM DTT, 0.1 mM EDTA, and 0.1 mM Fru-6-P, and 50 mM glycylglycine:KOH buffer (pH 7.5), containing 1 mM (NH4)2SO4 and 5 mM 2-mercaptoethanol, respectively. Apparent Kₘ values for each sample were measured under the above conditions at the indicated concentrations of MgATP. Velocities were usually expressed relative to the maximal velocity obtained after maximum stimulation with the activator or substrate of interest, unless otherwise indicated.

Kinetic assays were performed at the physiological pH (pH 7.35), since higher pH values decreased the allosteric interactions, while lower pH values yielded strong hysteretic responses which biased the interpretation of the results (data not shown). DTT and BSA were included to stabilize the enzyme.

**Differentiated and Anaplastic Thyroid Tumors of the Rat.** Medullary thyroid carcinomas were passaged in rats of the Wag/Rij strain by implantation of small sections (1 cu mm) of the tumor s.c. Originally, some rats of this strain spontaneously developed well-differentiated tumors which secreted large amounts of calcitonin and neuron-specific enolase (18, 25). These DMTCs were soft, well-vascularized, and slow-growing tumors which contained tubular structures and could be transplanted to other rats. After sequential transplantation, a deviated tumor line was obtained which showed an increase in growth rate and a decrease in peptide hormone and neuron-specific enolase production (25). Histologically, it was undifferentiated or AMTC with a fibrosarcomatous consistency and appearance. Following implantation, anaplastic and differentiated tumors reached an average size of approximately 5 g within 3 and 20 weeks, respectively, when they were surgically removed under ether anesthesia. The muscle, liver, and brain tissues were harvested from normal rats immediately after the sacrifice. The rat tumors and normal organs were stored or transported at −80°C; the studies were performed within 2 to 8 weeks of tissue collection.

**Preparation of Tissue Extracts.** For the chromatographic and immunological studies, fresh tissue extracts were prepared using the extraction buffer, 50 mM potassium phosphate (pH 8.0), containing 10 mM (NH4)2SO4, 1 mM EDTA, 0.5 mM Fru-6-P, 0.1 mM ATP, and 1.0 mM DTT as described previously (43).

**Partial Purification of the Various PFKs.** For the kinetic studies, PFKs from the tumors were partially purified, essentially according to the published procedures (8–10, 13, 39). Briefly, tumors dissected free of necrotic parts and hemorrhages were homogenized in 4 volumes of cold extraction buffer (pH 7.5), containing 0.2 mM Tris-phosphate; 10 mM (NH4)2SO4, 40 mM KF, 5 mM MgATP; 10 mM EDTA, 0.2 mM ATP, 0.1 mM Fru-6-P, 0.01 mM Fruc-1,6-P2, and 1 mM disopropylfluorophosphate. After removal of particulate matter, PFK was precipitated from the supernatant by (NH4)2SO4 (50 to 65% saturation), and subjected to heat treatment (58°C × 2 min) while suspended in 20 mM Tris-phosphate buffer (pH 7.5), containing 10 mM (NH4)2SO4, 10 mM KF, 5 mM MgATP, 0.1 mM EDTA, and 0.01 mM Fruc-1,6-P2. The heat-coagulated proteins were removed by centrifugation, and PFK was precipitated by (NH4)2SO4 (65% saturation). The enzyme was resuspended in a small volume of the latter buffer and stored frozen at −70°C until use. Recoveries of the enzyme were at least 90% at each of the (NH4)2SO4 fractionation steps and after heat treatment.

**Chromatographic Separation of PFK Isozymes.** PFK isozymes were resolved by using DEAE-Sephadex A-25 ion-exchange chromatography at 4°C as described previously (4, 46). Briefly, a 1.7 cm × 30 cm column was equilibrated in 0.1 mM Tris-phosphate buffer, (pH 8.0), containing 25 mM NaCl, 0.2 mM EDTA, 0.2 mM AMP, and 0.7 mM DTT. The column was loaded with 0.1 to 0.12 unit of the enzyme preparation unless otherwise indicated. A 300-ml concave elution gradient was prepared by using 3 chambers of a Varigrad 9-chambered gradient mixer, as described previously (4). A total of 100 3-ml fractions were collected and assayed for PFK activity. Extensive experience with the homogenization and chromatographic procedures indicate that PFK is stable throughout these procedures, since the recoveries of the enzyme activities are...
always quantitative.

Production and Characterization of Subunit-specific Antibodies. In order to undertake immunochemical analysis of the rat PFK isozymes, we chose to use the already available rabbit antibodies produced against human PFK isozymes (46, 47). The rabbit anti-muscle PFK antibody was produced using a homogeneous preparation of human muscle PFK, i.e., M4 isozyme (46). In contrast, anti-L antibody was raised against a partially purified preparation of human RBC PFK, i.e., a mixture of M4, M3L, M2L2, ML3, and L4 isozymes. Interestingly, one of the 3 rabbits given an injection of this preparation produced an antibody that reacted with only the L4 isozyme. It showed no reactivity with the muscle- and platelet-type homotetramers in the active enzyme immunoprecipitation assay described below. Both of these antibodies showed varying degrees of cross-reactivity with the various PFKs from the rat, as will be described elsewhere. The anti-M and anti-L antibodies were then extensively absorbed using non-M and non-L isozymes from the rat, respectively, to render them monospecific. The details of their reactivity patterns with the normal rat PFKs, prior to absorptions, and the techniques of absorption will be described elsewhere.

Active Enzyme Immunoprecipitation Assay. The anti-M and anti-L antibodies were diluted serially in phosphate-buffered saline to the highest dilutions of 1:1024 and 1:128, respectively, since, at these dilutions, the antibodies precipitated less than 10% of the respective PFK from the rat. Freshly prepared tissue extracts were diluted to a final concentration of 0.06 unit/ml with the extraction buffer described above. Samples (50 μl) of a given extract were mixed in duplicate with 50 μl of the diluted anti-M or anti-L antibodies; the duplicate control tubes contained non-immune rat serum at 1:4 dilution of phosphate-buffered saline. The precipitated enzyme activities were measured in 48,000 x g supernatants of tissue homogenates. Chart 1 illustrates the enzyme activity patterns of the various organs and tumors of the rat. A, a mixture of muscle (M4), brain (P4), and liver (L4) PFKs; B and C, isozymes from DMTC and AMTC, respectively; D and E, brain PFK isozymes when 0.05 and 0.025 units are loaded on the column; F, isozymes from DMTC, when only 0.025 unit is chromatographed.

and brain showed the intermediate PFK activity; the activities in DMTC were approximately 2-fold higher than those in AMTC. This difference was statistically significant (p < 0.01). The activities of HK and PK, although reported elsewhere (28), and G6PD were included here for the comparison. AMTC showed an increase in PK (about 2-fold) and a decrease in G6PD activities. In contrast, HK activities were not significantly different for the 2 tumor types.

Isozymes of PFK from the Rat Tumors. PFK activities were recovered quantitatively (95 ± 5% (S.D.) following the chromatography of the tissue homogenates. Chart 1 illustrates the representative isozymic profile of PFK from the normal organs and tumors of the rat. As reported elsewhere, the PFKs from rat muscle (M), brain (platelet or P4), and liver (L) (the predominant form) appear to mainly consist of 3 unique homotetramers, M4, P4, and L4, respectively. As shown in Chart 1A, muscle and brain PFKs elute as single peaks even when very small amounts of the enzyme, i.e., 0.08 unit, are chromatographed. A mixture of muscle and brain PFKs, when chromatographed, always shows the presence of 2 overlapping peaks. In contrast, although the rat liver PFK elutes as a major peak coincident with the human L4 tetramer, a few minor eluting species are generally present. The latter may represent the hybrids of muscle + liver or brain + liver or of all 3 subunits. The peaks of the muscle, brain, and liver isozymes elute at 260, 280, and 500 mOsmol/kg respectively, which correspond to approximately 47th, 55th, and 77th fraction, respectively, during chromatography. It is noteworthy that human M4, P4, and L4 tetramers elute at 214, 244, and 480 mOsmol/kg during the gradient elution (4), indicating that rat PFKs elute at the respective analogous positions.

As shown in Chart 1B, PFK from DMTC eluted mainly as a single peak at 325 mOsmol/kg; however, one or two minor species are visible towards the liver isozyme. The total span of the PFK isozymes from DMTC is from 275 to 470 mOsmol/kg.

### Table 1

<table>
<thead>
<tr>
<th>Organ or tumor</th>
<th>Enzyme specific activity (units/mg soluble protein)</th>
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<tbody>
<tr>
<td></td>
<td>PFK PK HK G6PD</td>
</tr>
<tr>
<td>Muscle</td>
<td>2.13 ± 0.40 0.78 ± 0.20 0.04 ± 0.01 0.11 ± 0.03 0.20 ± 0.07</td>
</tr>
<tr>
<td>Brain</td>
<td>8.5 ± 1.5  2.9 ± 0.6  0.17 ± 0.02  0.03 ± 0.01  1.9 ± 0.8</td>
</tr>
<tr>
<td>Liver</td>
<td>0.05 ± 0.02 0.17 ± 0.02 0.06 ± 0.02 0.06 ± 0.04 0.08 ± 0.04</td>
</tr>
<tr>
<td>AMTC</td>
<td>6.7 ± 0.20  5.0 ± 0.20  0.03 ± 0.01  0.04 ± 0.01  1.00 ± 0.05</td>
</tr>
<tr>
<td>DMTC</td>
<td>0.60 ± 0.05  0.05 ± 0.05  0.12 ± 0.04  0.12 ± 0.04  0.12 ± 0.04</td>
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* Mean ± S.D. of at least 4 and 8 determinations on each of the normal organs and tumors, respectively.
In contrast, the PFK from AMTC elutes as multiple isozymic species at 370, 400, and 430 mOsmol/kg, with the predominant form being liver-type in nature. The total span of the PFK isozymes from AMTC is much larger than that of DMTC, i.e., 280 to 550 mOsmol/kg, indicating an increase in the liver-type isozyme(s). It is noteworthy that both the tumor PFKs begin elution at ~280 mOsmol/kg, where the peak of the brain PFK elutes. These results suggest that tumor PFKs are largely composed of the brain- and liver-type subunits.

Chart 1, D and E, shows the profiles of the brain PFK, when it is chromatographed using 0.1 unit (standard amount) and 0.025 unit enzyme, respectively. The latter amount was expected to result in a better resolution of the isozymes, if any. In both instances, it elutes as a single peak at 280 mOsmol/kg, with a span of 235 to 360 mOsmol/kg, indicating that it consists of isolated P4 isozyme. Chart 1F shows the isozymes from DMTC, when chromatographed using 0.025 unit instead of 0.1 unit PFK. PFK from DMTC showed a major peak eluting at 325 mOsmol/kg and a minor species following it; both probably represent hybrids of P<sub>-</sub> and L-subunits.

Immunoochemical Analysis of the Tumor PFKs. Charts 2 and 3 show the immunochemical reactivity patterns of normal and tumor PFKs with absorbed anti-L and anti-M antibody, respectively. As shown in Chart 3A, the absorbed anti-L antibody is highly monospecific for the liver PFK; it shows no cross-reactivity with either muscle or brain PFKs. As shown in Chart 2B, PFKs from AMTC show a much greater degree of precipitation with anti-L antibody as compared to that from DMTC, indicating a greater expression of the liver-type subunit.

As shown in Chart 3A, the extensively absorbed anti-M antibody shows no reactivity with the liver-type PFK but continues to cross-react to a minor extent with the brain-type PFK, indicating a structural homology between the M- and P<sub>-</sub> subunits and, hence, "true-cross reactivity." This interpretation was borne out by the fact that continued absorption of anti-M antibody with the brain PFK resulted in the loss of reactivity with muscle PFK as well. Because of this persistent cross-reactivity, we determined the reactivity patterns of tumor PFKs using both unabsorbed and absorbed anti-M antibody. As shown in Chart 3B, the unabsorbed antibody reacts almost identically with the brain PFK and both of the tumor PFKs. These data confirm the chromatographic observation that tumor PFKs consist largely of the brain-type PFK. As expected, anti-M antibody extensively absorbed using brain PFK shows poor reactivity against both of the tumor PFKs (Chart 3C). These data, although noninformative...
from both tumors showed nonhyperbolic kinetics toward the substrate Fructose-6-P in the presence of different concentrations of MgATP. PFKs from the 2 tumor types showed no significant differences in substrate affinity. $S_{0.5}$ Fructose-6-P values of PFKs from both AMTCs and DMTCs ranged from 0.8 to 2.1 mM, when MgATP concentration was increased from 0.2 to 1.0 mM (data not shown).

Chart 4 illustrates the inhibition of tumor PFKs by the second substrate MgATP. At high Fructose-6-P concentrations (1.0 to 1.5 mM), the reaction velocity of PFKs from both the tumors showed an initial increase, which reached an optimum value at approximately 0.2 mM MgATP, followed by a gradual inhibition at increasing MgATP concentrations. At low Fructose-6-P concentrations (0.5 mM), PFK from DMTC showed a sharp activity peak with an optimum at 0.1 mM MgATP (Chart 4B), while PFK from AMTC maintained a low level of residual activity, even in the presence of relatively high MgATP concentrations (Chart 4A).

Regulatory Properties of Tumor PFKs. The effects of citrate, an inhibitor of PFK, and cAMP, an activator of the enzyme, were found to be as follows. As shown in Chart 5, PFKs from both the tumors were strongly inhibited by citrate; the level of inhibition was dependent, to a large extent, on the pH of the reaction medium and the concentrations of MgATP. Fructose-6-P, and free Mg$^{2+}$ ions (data not shown). Interestingly, the susceptibility of PFK from AMTC to citrate inhibition was always lesser than that from DMTC, apparent $K_v$ values for the former being twice that for the latter. The reversal of citrate inhibition by cAMP was more effective for PFK from AMTC than from DMTC. This activation or deactivation was more pronounced at citrate concentrations greater than 0.5 mM. For instance, 0.2 mM cAMP reactivated the citrate (2.0 mM)-inhibited activity from AMTC and DMTC by 26 and 9%, respectively, of the fully uninhibited value; i.e., cAMP induced velocity in the absence of citrate (data not shown). Thus, in the presence of inhibiting concentrations of citrate, cAMP reactivated the former enzyme to a much higher extent than was to be expected based solely on the small difference in sensitivity towards the inhibitor.

Activating Effect of Fructose-2,6-P$_2$ on Tumor PFKs. The activating effect of Fructose-2,6-P$_2$, as well as its ability to deinhibit the inhibition by citrate and/or MgATP, was also evaluated. Chart 6 illustrates that both tumor PFKs were strongly stimulated by Fructose-2,6-P$_2$; however, the extent of activation differed. The half-maximal activation of PFK from AMTC occurred at 6-fold lower Fructose-2,6-P$_2$ concentrations than that required for the half-maximal activation of PFK from DMTC; the $K_v$ values for the PFKs from AMTC and DMTC were 0.5 and 3 mM, respectively.

As shown in Chart 7, reversal of citrate-inhibition of PFK from AMTC by Fructose-2,6-P$_2$ was much more effective compared to that of PFK from DMTC. At citrate concentrations up to 6 mM, the inhibition of the enzyme from AMTC could be reversed by 0.1 mM Fructose-2,6-P$_2$ (i.e., saturating concentration) to more than 50% of its fully deinhibited activity. Under identical conditions, the reversal of the inhibition of the enzyme from DMTC was almost negligible. These findings therefore suggest a greater cooperative interaction of citrate-inhibited PFK from AMTC with Fructose-2,6-P$_2$ than PFK from DMTC, since the difference in kinetic behavior of the 2 tumor PFKs toward the combined effects of citrate and Fructose-2,6-P$_2$ is far more pronounced than toward the inhibitor or activator alone. In addition, activation of citrate-inhibited PFKs showed a marked dependence on the concentration of simultaneously added MgATP. In the absence of citrate and at saturating Fructose-2,6-P$_2$ concentration, only marginal differences were observed between tumor PFK activity at any given MgATP concentration. On addition of increasing amounts of citrate, these differences gradually increased. At 5.0 mM citrate concentration, PFK from AMTC could be reactivated by 0.1 mM Fructose-2,6-P$_2$ to approximately 90 and 80% of its control level in the presence of 0.5 and 4.0 mM MgATP, respectively, while, for PFKs from DMTC, these values were 70 and 30% (data not shown), thus indicating again that PFK from AMTC is more sensitive to reversible inhibition of stress than is PFK from DMTC.

Intratumoral Content of Fructose-2,6-P$_2$. In order to examine whether the increased sensitivity of PFK from AMTC to the activation by Fructose-2,6-P$_2$ is attended with increased intratumoral levels of the compound as compared to DMTC, we measured Fructose-2,6-P$_2$ in NaOH extracts of the tumors by its ability to stimulate pyrophosphate:Fructose-6-P phosphotransferase from potato tubers (42). However, Fructose-2,6-P$_2$ concentrations assayed in this way were extremely low (mean for both tumors, 0.5 ± 0.2 nmol/g, wet weight). It is conceivable that, due to the extremely
rapid turnover of Fru-2,6-P₂ (11, 26, 40), its actual levels in vivo at different stages of tumor development are higher than observed here.

DISCUSSION

Previous studies of the experimental rodent tumors and human cancers have shown that PFK exhibits both quantitative alterations in activity and qualitative isozymic shifts in neoplasia (7, 30, 44, 45, 48). In transplantable rat hepatomas, PFK showed a 2- to 3-fold increase in poorly differentiated (rapidly growing) tumors, while it was normal in well-differentiated (slowly growing) tumors (7), indicating a direct correlation of the enzyme activity with the degree of differentiation (6-8, 16). This increase was also associated with altered kinetic and regulatory properties of the tumor enzyme (7, 34) and was shown to result from an increase in the liver-type isozyme (7, 16, 17, 33) and from the appearance of new isozymes. The number and nature of the new species have remained controversial (5, 8, 17). The latter may be largely attributable to the differences in the strains studied and rates of growth of tumors, as well as techniques used to demonstrate the isozymes.

Studies of the nonmedullary thyroid tumors of the rat by Meldolesi and Lacetti (22), Meldolesi and Maccia (23), and Meldolesi et al. (24) showed a lack of rate-limiting activity of tumor PFK as compared to the normal thyroid enzyme. This property was accompanied by altered kinetic and chromatographic behavior of the tumor PFK (22-24). Although the tumor PFK later was found to be more acidic as compared to the normal thyroid PFK, the precise isozymic nature of both enzymes were completely unknown (22, 24).

Studies of various human cancers and cultured cell lines have shown that human PFK also exhibits comparable changes in activity and isozymes (for review, see Ref. 45). The increases in PFK activity are correlated with the rates of replication of the malignant cell types, and the isozymic alterations parallel the quantitative increases in the total enzyme activity; i.e., the higher the increment, the more dramatic are the isozymic alterations (1, 3, 43, 45). In general, PFK exhibits quantitative increases similar to those exhibited by PK and HK in neoplasia. However, unlike the isozymic alterations in HK and PK, where the highly regulated liver-type isozymes are largely replaced by the nonregulated ones (29, 50), in the case of PFK, the liver-type isozyme not only persists but actually increases.

The results of the present study dramatically reinforce the preferential expression of the liver-type isozyme by cancer cells. The DMTCs of the rat show largely brain-type isozyme, with a minor amount of the hybrid isozyme(s) of brain and liver-type subunits both chromatographically and immunologically (Charts 1C and 2B). Although the AMTCs also express multiple isozymes...
PFK ISOZYMES IN MEDULLARY THYROID CARCINOMAS OF THE RAT

Chart 7. Reversal of citrate inhibition of tumor PFKs by Fru-2,6-P2. Tumor PFKs were inhibited by increasing concentrations of citrate in the presence of 1.0 mM MgATP, 1.0 mM Fru-6-P, and 1.5 mM Mg++. Reactions were started with an amount of sample that would give a rate of change of absorbance of approximately 0.020 per min at 340 nm in the absence of citrate. At citrate concentrations >0.5 mM, no activity was detected in the absence of Fru-2,6-P2. Reversal of citrate inhibition of PFKs from AMTC (■) and DMTC (▲) by 0.1 mM Fru-2,6-P2 was measured in the same cuvet by the addition of the compound. % v/vm, percentages of maximal velocities.

of brain and liver-type subunits, the L-subunit-containing isozyme(s) predominate in these tumors (Charts 1C and 2B). Due to the persistent cross-reactivity of anti-M antibody with the brain-type PFK, it is unclear whether the M-subunit is present as a minor isozyme and is differentially expressed by these tumors or not. Since medullary thyroid carcinomas originate from the parafollicular C-cells and not from the epithelium lining the thyroid ducts, the PFK profiles of these tumors cannot be compared with those of normal thyroid gland and other experimental thyroid tumors, which are mainly comprised of ductular epithelial cells.

The observation that AMTCs show a decrease in total PFK activity as compared to DMTCs is inconsistent with the fact that rapidly growing rat hepatomas and thyroid carcinomas show impressive increases in activities of not only PFK but also PK and HK (22, 30). The simultaneous increases in PK activity and decreases in 6PD activity in AMTCs are indeed suggestive of a greater dependence on glycolysis as compared to hexose monophosphate shunt by these tumors. A decrease in PFK activity appears incongruous, since these tumors exhibit higher rates of aerobic glycolysis in vivo, as indicated by higher levels of lactic acid both in tumors and plasmas of tumor-bearing animals (28). It is conceivable that PFK activity may be increased by metabolic regulation in these tumors. In order to elucidate such regulatory mechanism(s), if any, we investigated the kinetic and regulatory properties of the tumor PFKs.

Both tumor PFKs showed highly allosteric kinetics toward Fru-6-P, and their apparent So values for Fru-6-P were essentially identical. Meldolesi et al. (24) also reported highly cooperative kinetics with Fru-6-P for both normal thyroid and tumor enzymes. These data, taken together with those reported for rabbit brain PFK (35), which also exhibits allosteric kinetics, cast several doubts on the reportedly poor allosteric nature of platelet or brain-type PFK (20).

Despite identical So values for Fru-6-P, the 2 tumor PFKs demonstrated differential inhibition by citrate and MgATP and activation by Fru-2,6-P2. PFK from AMTCs was less sensitive to inhibition by citrate than that from DMTC (Chart 4). In addition, reactivation of the citrate-inhibited enzyme (deinhibition) by cAMP, a potent allosteric activator, was considerably more effective for the enzyme from AMTC than that from DMTC. These observations could be partly accounted for by the fact that liver isozyme is less sensitive to inhibition and more sensitive to activation by citrate and cAMP respectively, as compared to the 2 nonliver isozymes (34). PFK from AMTCs was less inhibitable by MgATP than was that from DMTC only at low Fru-6-P concentrations (Chart 5). These results are inconsistent with the well-established fact that, among the 3 PFK isozymes, liver-type isozyme is the most sensitive to ATP inhibition. It is possible that the simultaneous presence of the brain-type isozyme is responsible for this difference. A decrease in the allosteric nature of the tumor isozymes has been reported previously (7, 24, 32).

Both tumor PFKs were activated by Fru-2,6-P2. However, consistent with the observed increase in the liver-type isozyme in AMTC, the enzyme from AMTC was more sensitive to activation by Fru-2,6-P2 after inhibition by both citrate and MgATP (Chart 7); apparent Kf Fru-2,6-P2 values for AMTC and DMTC were 0.5 and 3 μM, respectively. Citrate-inhibited PFK from DMTCs (mainly brain-type) was less sensitive to activation by cAMP than was PFK from AMTC. These results differ from those reported for the rabbit brain PFK, which is sensitive to activation by cAMP to the same extent as the muscle and liver type isozymes (37). The reason for this discrepancy may lie in the fact that the rabbit brain PFK consists of a 5-membered isozyme set composed of the M- and P4-subunits (37). In contrast, PFKs from rat brain and DMTC consist mainly of brain-type and brain-plus type minor amount of liver subunits, respectively. Differential phosphorylation states of the tumor PFKs may be invoked to explain their different regulatory properties (9, 10, 13, 31). However, the role of phosphorylation in modulating kinetics of PFK has been questioned recently (27).

It is tempting to speculate on the physiological relevance of the differential affinities of the tumor PFKs or those of brain and liver PFKs for Fru-2,6-P2. In liver and cultured hepatocytes, an increase in Fru-2,6-P2 is associated with an increased rate of glycolysis, suggesting a critical role for this compound in the regulation of glycolysis in this organ (10, 26, 40); however, recent studies question its role (12, 40). It is quite conceivable that Fru-2,6-P2 assumes a special regulatory role in the cancer cell in that aerobic glycolysis is enhanced. Dunaway et al. (6) reported a 2- to 3-fold increase in the so-called "regulatory factor" for PFK in poorly differentiated rat hepatomas as compared to normal liver. Recently, Van Schaftingen and Hers (41) have compared this regulatory factor with Fru-2,6-P2 and have suggested that these may be identical. Although the levels of Fru-2,6-P2 were found to be essentially identical in the extracts of both types of tumors, it is conceivable that its very low levels in vivo coupled with an extremely rapid turnover which is also under the hormonal and dietary influences (11, 26, 40) do not permit a precise assessment of its in vivo concentrations. Our preliminary data using partially purified PFKs from the rat suggest that the liver isozyme is the most sensitive to activation by Fru-2,6-P2 and, thus, at the identical intratumoral levels of the compound, the enzyme from AMTCs could be more active as compared to that from DMTCs. Although our data are inconsistent with the previous observation that muscle PFK has a higher affinity for the com-
the affinity of PFK isozymes for Fru-2,6-P2 could account for this discrepancy.

In summary, the AMTCs from the rabbit exhibit a decrease in PFK activity as compared to DMTCs accompanied by an increase in the expression of the liver-type isozyme. Notable among these alterations is the increased sensitivity of the enzyme from AMTCs to citrate and the increased sensitivity to activation by Fru-2,6-P2. The latter may explain the enhanced glycolytic rates of these tumors despite decreased PFK activity.

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