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THE MONOMER OF PYRUVATE KINASE, SUBTYPE M_1, IS BOTH A KINASE AND A CYTOSOLIC THYROID HORMONE BINDING PROTEIN

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Using a T7 expression system, the monomer of rat pituitary pyruvate kinase, subtype M_1 (PKM_1), was overexpressed in *Escherichia coli* and purified to homogeneity. The monomeric p58-M_1 has intrinsic enzymatic activity with a Vmax of 79 ± 20 units/mg and Km's for ADP and PEP of 1.43 ± 0.76 and 0.14 ± 0.07 mM, respectively. The monomer binds 3,3',5-triiodo-L-thyronine (T_3) with Ka = 1.5 x 10^7 M^{-1}. The order of analog specificity is L-T_3 > L-thyroxine > D-T_3 > 3'-isopropyl-3,5-diiodo-L-thyronine ≥ 3',5',3-triiodo-L-thyronine. In contrast, tetrameric PKM_1 lacks T_3 binding activity. The kinase activity of p58-M_1 is inhibited by T_3 and its analogs in a concentration-dependent manner with the order of inhibitory activity similar to that of binding activity. This inhibition, however, is reversed by the addition of fructose 1,6-bisphosphate. p58-M_1 is the second PK isoenzyme monomer to be identified as having thyroid hormone binding activity.

Introduction - Cytosolic thyroid hormone binding protein (CTHBP) is widely present in tissues of many species and cultured cells (1). However, the binding affinity for T_3 varied greatly from 6.6 x 10^6 M^{-1} for CTHBP from rat cerebellum (2) to 3 x 10^9 M^{-1} for CTHBP from human red blood cells (3). It is unclear, however, whether these variations reflect differences in the cytosolic preparations under different experimental conditions or differences in the identity of protein molecules from different species and tissues. Furthermore, in rat brain, liver and heart, CTHBP was found to be developmentally regulated (2,4). It is unknown whether the changes reflect the differential regulation of different CTHBPs at different stages of development.

We have purified a human CTHBP to homogeneity from human epidermoid carcinoma A431 cells (5). It has a molecular weight of 58,000 and consists of a single polypeptide chain (p58-M_2). Sequence analysis of p58-M_1 cDNA indicated that p58-M_2 is homologous to a subunit of pyruvate kinase, subtype M_2 (PKM_2) (6). Pyruvate kinase (ATP:pyruvate O^2-phospho-

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Abbreviations: T_3, 3,3',5-triiodo-L-thyronine; CTHBP, cytosolic thyroid hormone binding protein; Fru-1,6-P_2, fructose 1,6-bisphosphate; PK, pyruvate kinase; T_4, L-thyroxine; 3'-Isopropyl-T_2, 3'-isopropyl-3,5-diiodo-L-thyronine; r-T_3, 3',5',3-triiodo-L-thyronine; PEP, phosphoenol pyruvate; ADP, adenosine diphosphate.

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transferase, EC 2.7.1.40) is an important glycolytic enzyme which is expressed and regulated in a tissue specific manner. There are four mammalian isoenzymes of PK known, each of which consists of four identical or nearly identical subunits with the molecular weight of each subunit ranging from 57-60K (7). The four isoenzymes are designated as L, R, M_1 and M_2. L-type is mostly present in liver; R type is found exclusively in erythrocytes; M_1 type is mostly in muscle and M_2 type is widely found in many tissues such as kidney, intestine, lung, fibroblasts, testis, adipose tissue and stomach. PKM_1 and PKM_2 arise as a result of alternative splicing of a precursor mRNA (8). PKM_1 is reported not to be activated by Fru-1,6-P_2, in contrast to PKM_2 (7).

Based on the above consideration, it is possible that the heterogeneity observed for the interaction of T_3 with CTHBP in different tissues and in different developmental stages could be due to the existence of different T_3 binding molecular species and to their different modes of regulation. However, purification of monomeric PKM_1 in its native form has not been reported. Its kinase and T_3 binding characteristics are unknown. In the present study, we adopted the strategy of recombinant techniques to overexpress the monomeric PKM_1 in *E. coli* and purified it to homogeneity. We investigated the T_3 binding activity of p58-M_1 and the effect of thyroid hormones and Fru-1,6-P_2 on its enzymatic properties.

Materials and Methods

[3'-^{125}I]T_3 (2200 Ci/mmole) was obtained from DuPont New England Nuclear. Fru-1,6-P_2, PEP, ADP and NADH, lactate dehydrogenase, 2,4-dinitrophenylhydrazine, L-T_4, D-T_3, r-T_3, T_3 and rabbit pyruvate kinase were from Sigma.

Construction of the expression plasmid pCJ22: The expression vector, pCJ3, which contains the coding region of human placental c-*erb*A was restricted with Nde I and EcoR I. The insert which contains the coding region of pGH35 (9) which encodes the pyruvate kinase from GH_3 cells was prepared by polymerase chain reaction using pGH35 as a template and ligated into the vector similarly as described (10).

Expression and purification of p58-M_1: The expression of p58-M_1 in BL21/LysS cells and subsequent purification were carried out similarly as described by Ashizawa *et al.* (10) with the following modifications. After the Q-Sepharose column chromatograpy, the flow-through was applied to a S-Sepharose column (1.2 x 9 cm). After washing the S-Sepharose column with 20 ml of buffer S, p58-M_1 was eluted with a 20 ml linear gradient from 0 to 50 mM NaCl in buffer G (100 mM PO_4, 1 mM EDTA, 0.1 mM phenylmethylsulfonyl fluoride pH 6.0) followed by a 20 ml wash with 50 mM NaCl in buffer G and 2 ml fractions were collected. p58-M_1 was eluted at 10 mM NaCl. The purity of the protein was analyzed by 10% SDS-PAGE.

Determination of the molecular size of the purified p58-M_1: The molecular size of the purified p58-M_1 was analyzed by a TSK gel (G3000 SW) column (7.5 mm x 60 cm) using a fast pressure liquid chromatography system (FPLC, Pharmacia Co.). Purified proteins (~0.1 - 0.2 mg/1 ml) were loaded onto the column. The proteins were fractionated with a flow rate of 0.6 ml/min in buffer F (50 mM Tris, 0.1 M KCl, 5 mM PO_4, pH 7.1) with or without 5 mM Fru-1,6-P_2 and 0.25 ml/fraction were collected. Each fraction was analyzed for pyruvate kinase and T_3 binding activity. The column was calibrated with bovine serum albumin and rabbit pyruvate kinase.

Enzymatic activity: Pyruvate kinase activities were measured using the NADH-LDH coupled assay described by Imamura and Tanaka (7), with the exception that Fru-1,6-P_2 was normally omitted from the mixture. Analysis of data were carried similarly as described in (10).

Binding of [^{125}I]T_3 to p58-M_1: Binding of [^{125}I]T_3 was carried out by incubation of p58-M_1 with 0.5 nM [^{125}I]T_3 in the presence or absence of competitors (1-1000 nM) for 1 hr at 4°C. The protein-bound [^{125}I]T_3 was separated from unbound radioligand on a Sephadex G-25 (fine) column (5).

Results

Expression and purification of p58-M_1: The expression and purification of p58-M_1 was analyzed by SDS-PAGE. Lane 1 of Fig. 1A shows the protein pattern from *E. coli* lysate which has not been transformed by the expression vector pCJ22. Lane 2 of Fig. 1A shows that a new major protein with a molecular weight of 58,000 was detected in the cell lysate containing the expression vector. Analysis of the purity of p58-M_1 after Q-Sepharose column chromatography indicated that the protein was pure (lane 4, Fig. 1A). In some experiments, a few trace contaminants (<0.5%) with molecular weight lower than 55,000 were detected by silver staining. If this was the case, we further purified p58-M_1 by S-Sepharose column chromatography (lane 5, Fig. 1A). Approximately 3 mg of purified protein was obtained from one liter of *E. coli* culture.

Panel B in Fig. 1 shows that the expressed p58-M_1 was recognized by J11, a monoclonal antibody raised against p58-M_2, (lane 2 of Fig. 1B) (11). In contrast, no protein in *E. coli* lysate was recognized by J11 as shown in lane 1. Lanes 2-5 show that at each stage of the purification step, p58-M_1 retained its immunoreactivity.

Association of the purified monomeric p58-M_1 to form tetrameric pyruvate kinase (PKM_1): Previously, we have shown that in the absence of Fru-1,6-P_2, the purified p58-M_2 existed as a monomer (6,10). To understand whether the association of p58-M_1 to form tetrameric PKM_1 is also similarly regulated by Fru-1,6-P_2, we first evaluated the molecular size of the purified p58-M_1 by a TSK gel sizing column in the absence or presence of Fru-1,6-P_2. In the absence of Fru-1,6-P_2, Figure 2A shows that the purified protein consisted of two molecular species. One eluted at the position identical to the standard pyruvate kinase and another eluted with a elution position slightly behind that of bovine serum albumin. Characterization of the high molecular species showed that it had the enzymatic characteristics of PKM_1 (see Fig. 2). However, it lacked T_3 binding activity. The low molecular weight species had T_3 binding activity.

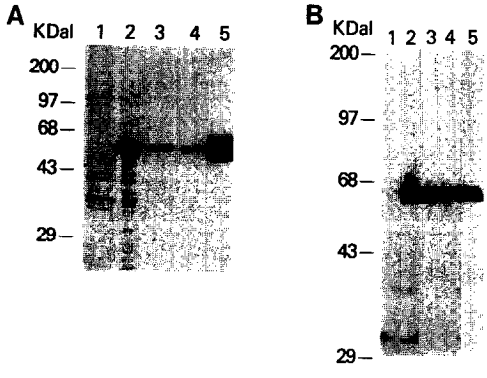


Figure 1. Analysis of the purity of p58-M_1 at each step of purification by SDS-PAGE (A) and Western blotting (B): An aliquot of samples at each step of purification was loaded onto a 10% gel and stained with Coomassie Blue (lanes 1-5 in Panel A). In Panel B, in a separate gel, the proteins were blotted onto nitrocellulose paper and reacted with monoclonal antibody J11 according to Obata *et al.* (11). Lane 1, lysate (10 μg) from BL21/Lys cells; lane 2, lysate (10 μg) from BL21/LysS cells transformed by pCJ22; lane 3, solubilized p58-M_1 (3 μg); lane 4, Q-Sepharose column flow-through (3 μg);and lane 5, p58-M_1 (10 μg) after S-Sepharose column chromatography. For Western blotting (B) the lanes are the same as in Panel A except that the amounts of proteins loaded onto the gel for each lane were approximately 0.5 μg.

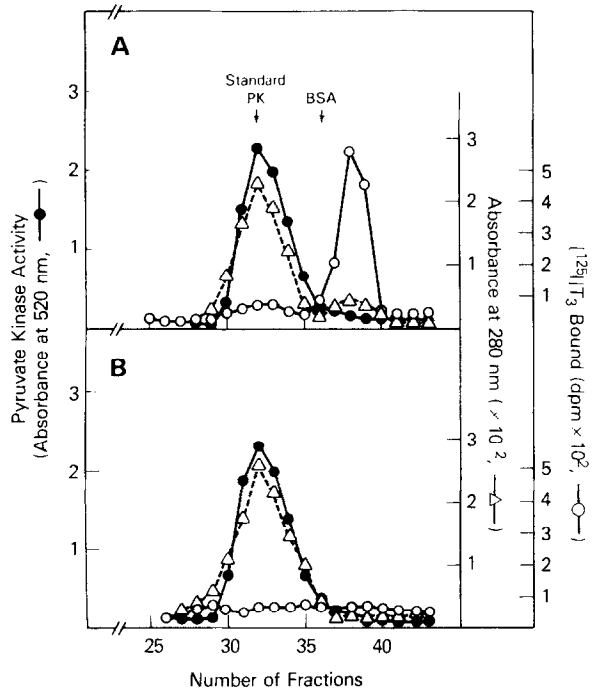


Figure 2. Characterization of the molecular size of the expressed p58-M_1 by a TSK gel column: One ml of purified p58-M_1 (132 μg/ml) obtained after Q-Sepharose column chromatography was applied to a TSK gel column (0.75 x 60 cm) attached to a FPLC system. The protein was fractionated in a buffer containing 50 mM Tris/7.1, 100 mM KCl and 5 mM Mg_2SO_4 in the absence (Panel A) or presence (Panel B) of 5 mM Fru-1,6-P_2. The protein was fractionated with a flow rate of 0.6 ml/min and 0.25 ml/fraction were collected. Aliquots were analyzed for [^{125}I]T_3 binding (○) and absorbance at 280 nm (Δ). PK activity was determined (●) by the 2,4-dinitropheylhydrazone method as described (7).

To estimate the relative amounts of the tetrameric and monomeric form, fractions 29-45 from TSK gel column chromatography were analyzed by SDS-PAGE and Western blotting using J11. The monomeric p58-M_1 was estimated to be ~15-20% in the purified preparation. Thus, both monomeric p58-M_1 and tetrameric PKM_1 were present under the experimental conditions.

Similar to p58-M_2, Fru-1,6-P_2 facilitated the formation of the monomeric p58-M_1 to the tetrameric PK. As shown in Fig. 2B, in the presence of 5 mM Fru-1,6-P_2, the monomeric p58-M_1 was converted into the tetrameric PK as indicated by the disappearance of T_3 binding activity.

The monomeric p58-M_1 binds thyroid hormones: As shown in Fig. 2A, the monomeric p58-M_1 binds T_3. Its thyroid hormone binding characteristics were further evaluated. Figure 3 shows the results of competitive binding of T_3 and its analogs to p58-M_1. The binding constant was calculated to be $1.5 \pm 0.5 \times 10^7$ M^{-1} (mean ± standard deviation, n=3). Figure 3 further shows that L-T_4 and D-T_3 bound to p58-M_1 with 25% and less than 1% of the activity of T_3, respectively. 3'-Isopropyl-T_2 and r-T_3 have little or no binding activity.

The pyruvate kinase activity of p58-M_1 and PKM_1: Fractionation by the sizing column indicated that p58-M_1 purified from *E. coli* lysate associated to form a tetramer. To understand the enzymatic activities of the recombinant PKM_1, we evaluated its kinetic characteristics. The fraction corresponding to the tetramer from the sizing column (see Fig. 2A)

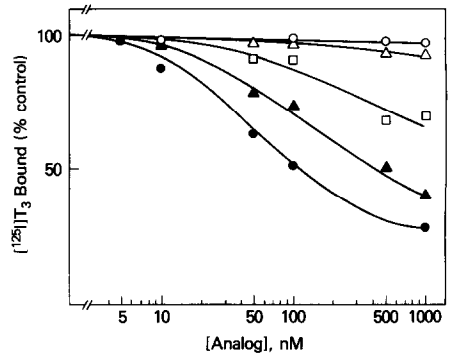


Figure 3. Competitive binding of L-T_3 and its analogs to p58-M_1: p58-M_1 (0.56 µg/0.28 ml) was incubated with 0.5 nM [^{125}I]T_3 in the absence or in the presence of increasing concentrations of unlabeled T_3 (●), L-T_4 (▲), D-T_3 (❑), 3'-Isopropyl-T_2 (Δ) or r-T_3 (o) for 1 hr. at 4°C. The free and bound [^{125}I]T_3 was separated by a G-25/fine column (5).

was analyzed. The kinetic data shown in Panel I of Fig. 4 were fitted to the Michaelis-Menten equation. The isolated tetramer had a maximal specific activity (V_{max}) of 204 ± 23 units/mg (Table I). The Km's for ADP and PEP are 0.52 ± 0.19 and 0.046 ± 0.015 mM, respectively. These values are similar to those described for PKM_1 isolated from rat (7). We further evaluated whether the enzymatic activity of the tetramer was enhanced by preincubation with Fru-1,6-P_2. As shown in Table I, within experimental error, Fru-1,6-P_2 did not change significantly either the V_{max} or Km's for ADP and PEP of PKM_1. Previously, it has been shown that Fru-1,6-P_2 did not increase the activity of PKM_1 isolated from rat tissues (7).

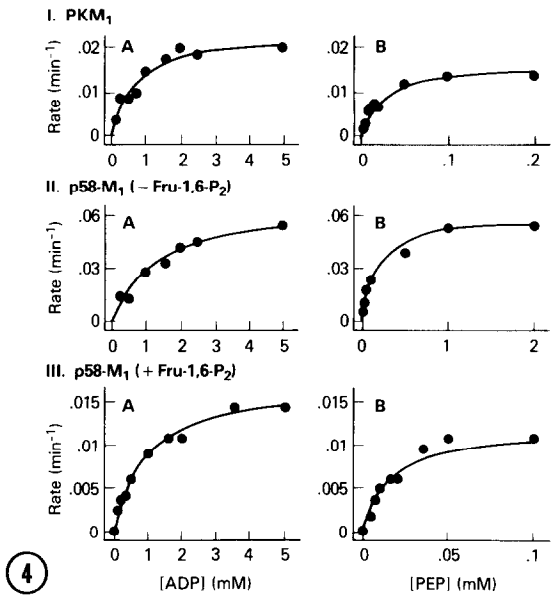
To address the question of whether p58-M_1 has intrinsic kinase activity, we also evaluated the kinetic properties of the isolated p58-M_1. Interestingly, kinase activity was detected. However, its kinetic characteristics are distinct from those of PKM_1. It has a lower V_{max} of 79 ± 20 units/mg. The Km's for ADP and PEP are significantly higher than those of PKM_1 (Fig. 4 and Table I). Upon incubation with Fru-1,6-P_2, however, the kinetic parameters were changed to values which were characteristic of the tetramer. These results indicated that monomeric p58-M_1 has intrinsic kinase activity. Furthermore, Fru-1,6-P_2 facilitates the association of p58-M_1 to become tetrameric PKM_1. These findings are consistent with the results shown in Fig. 2.

Since p58-M_1 binds T_3 and its analogs, we examined the effects of T_3 on the kinase activity of p58-M_1. As shown in Fig. 5, the enzymatic activity of p58-M_1 was inhibited by T_3 and

Table I. Kinetic Characteristics of Pyruvate Kinase Activities of p58-M_1 Under Various Conditions[a]

Enzyme Form	Fru-1,6-P_2 (2 mM)	Km (mM) ADP	Km (mM) PEP	V_{max} (units/mg)
PKM_1	-	0.52 ± 0.19	0.046 ± 0.015	204 ± 23
	+	0.70 ± 0.17	0.025 ± 0.005	217 ± 13
p58-M_1	-	1.43 ± 0.56	0.14 ± 0.07	79 ± 20
	+	0.74 ± 0.15	0.015 ± 0.002	200 ± 11

[a]The measurements were made at 25°C using NADH-LDH coupled assay (7).



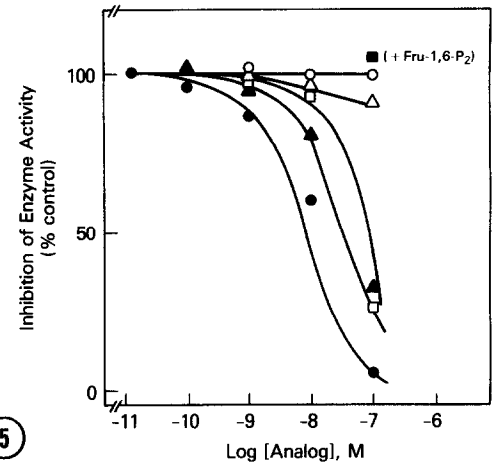


Figure 4. Effect of substrate concentrations on the pyruvate kinase activity of the purified PKM_1 and p58-M_1 after TSK gel column chromatography: I. PKM_1 (0.01 μg/ml) was assayed for its enzymatic activity in the presence of 2 mM PEP with variation of ADP concentration (A) or for its enzymatic activity in the presence of ADP in the variation of PEP concentration (B). II and III. p58-M_1 was assayed for its enzymatic activity in the absence (Panel II) or presence (Panel III) of Fru-1,6-P_2. Variation of PEP and ADP was similar to those described in Panel I. The concentration of p58-M_1 in (II) was 0.04 μg/ml and in (III) was 0.01 μg/ml.

Figure 5. Concentration-dependent inhibition of pyruvate kinase activity of p58-M_1 by thyroid hormones: Purified p58-M_1 was preincubated with increasing concentrations of T_3 (●), T4 (▲), D-T_3 (❑) 3'-Isopropyl-T_2 (Δ) and r-T_3 (○) for 2 hours at 4°C. An aliquot (100 ng/0.25 ml) was assayed under standard conditions (2 mM PEP, 2 mM ADP) using an NADH-LDH coupled assay as described in (7). ■ represents the enzymatic activity when 2 mM of Fru-1,6-P_2 was present in the preincubation mixture.

its analogs in a concentration-dependent manner. The order of the inhibitory activity of the analogs was parallel to that of binding activity (L-T_3>L-T_4>D-T_3>3'-isopropyl-T_2≥r-T_3). This inhibition was reversible. Preincubation of p58-M_1 with Fru-1,6-P_2 eliminated the inhibitory activity of thyroid hormones.

Discussion

Using a T7 expression system, large amounts of p58-M_1 was expressed in *E. coli*. Analysis of the molecular size of the purified protein by SDS-PAGE showed one protein band with an apparent molecular weight of 58,000. However, analysis of the purified protein by a sizing column showed that the purified protein consisted of two components. The majority (~80-85%) is the tetrameric PKM_1 which has a molecular weight of ~250,000. The minor component (~15-20%) exists as a monomer with a molecular weight of 58,000. Kinetic characterization of the PKM_1 showed that its enzymatic properties are similar to those described for rat PKM_1 obtained from tissues (7). This indicates to us that the recombinant p58-M_1 retains the native structure as it forms the tetrameric PK. When separated from the tetrameric form by gel filtration, the monomer

proved to have low enzymic activity, which was greatly enhanced by preincubation with Fru-1,6-P_2 due to the formation of tetramer. This is similar to our previous observations with PKM_2 (6,10). Likewise, the enzymatic activity of p58-M_1 was inhibited by preincubation with L-T_3. This inhibition was prevented by the inclusion of Fru-1,6-P_2 in the preincubation mixture. In both cases, this results from conversion of monomer to tetramer by the Fru-1,6-P_2. Thus, PKM_1 is the second isoenzyme of the PK multigene family for which it has been shown that monomer to tetramer conversion is regulated by Fru-1,6-P_2.

Of great interest is that the monomer binds T_3 and exhibits analog specificity. CTHBP has been shown to act as an intracellular transporter for thyroid hormones. Evidence has been presented to indicate that these proteins mediate the uptake of T_3 into mitochondria (12) and nuclei (13). Furthermore, p58-M_2 has been postulated to be involved in the metabolic effects induced by thyroid hormone (6). Even though its precise mechanism is unknown at the present time, the findings that similar to p58-M_2, p58-M_1 also binds T_3 raised the possibility that CTHBP's are members of a multigene family. They are expressed in a tissue-specific manner and are developmentally regulated. Thus, the requirement of CTHBP for mediating T_3 effects can be met at different stages of development in a tissue specific manner based on the specific signals and demands of cells.

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