An in Vitro Novel Mechanism of Regulating the Activity of Pyruvate Kinase M_2 by Thyroid Hormone and Fructose 1,6-Bisphosphate

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ABSTRACT: We have recently shown that the cytosolic thyroid hormone binding protein (p58-M_2) in human epidermoid carcinoma A431 cells is a monomer of pyruvate kinase, subtype M_2 (PKM_2). To characterize further the molecular properties of p58-M_2, we overexpressed p58-M_2 in *Escherichia coli* and purified it to homogeneity. At 22 °C, the monomeric p58-M_2 exhibited kinase activity with an apparent V_{max} of 22 ± 9 units/mg. The K_m for adenosine diphosphate (ADP) and phosphoenolpyruvate (PEP) are 3.85 ± 2.4 and 1.55 ± 0.73 mM, respectively. Upon activation by fructose 1,6-bisphosphate (Fru-1,6-P_2), V_{max} and K_m for ADP and PEP were changed to 490 ± 27 units/mg and 0.63 ± 0.09 and 0.13 ± 0.01 mM, respectively. These results indicated that p58-M_2 has intrinsic kinase activity. Analysis of the molecular size indicated that the activation of p58-M_2 by Fru-1,6-P_2 resulted in the association of the monomeric p58-M_2 to the tetrameric PKM_2. p58-M_2 bound to 3,3′,5-triiodo-L-thyronine (T_3) ($K_a = 1.7 \times 10^7$ M^{-1}) and exhibited analogue specificity, whereas PKM_2 did not bind thyroid hormone. The order of binding affinity was L-T_3 > L-thyroxine > 3,3′,5-triiodothyropropionic acid > 3′-isopropyl-3,5-triiodo-L-thyronine > 3′,5′,3-triiodo-L-thyronine. Binding of T_3 and its analogues resulted in the inhibition of the kinase activity of p58-M_2. The order of kinase inhibitory activity and preventing its association to tetrameric PKM_2 was parallel to that of binding activity. The present study demonstrated that, in vitro, the molecular mechanism by which Fru-1,6-P_2 induced activation of PK activity is by tetramer formation. Furthermore, thyroid hormone plays a role in regulating the enzymatic activity of PKM_2.

Pyruvate kinase (PK;[1] ATP:pyruvate O^2-phosphotransferase, EC 2.7.1.40) is an important glycolytic enzyme which catalyzes the conversion of phosphoenolpyruvate to pyruvate with the generation of ATP. There are four mammalian isoenzymes of pyruvate kinase known, each of which consists of four identical or nearly identical subunits, with the molecular weight of each subunit ranging from 57K to 60K (Imamura & Tanaka, 1982). The four isoenzymes are designated as L, R, M_1, and M_2. They are expressed in a tissue-specific manner. 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_2 is increased in regenerating liver and liver of tumor-bearing animals. The L and R isozymes differ from the M_1 and M_2 forms in their electrophoretic, kinetic, and immunological properties. cDNAs and genomic clones for rat L, R, M_1, and M_2 isoenzymes have been isolated and sequenced (Noguchi et al., 1986, 1987). The L- and R-type and the M_1- and M_2-type isozymes are under the control of different genes. The L and R types of rat pyruvate kinase are produced from a single gene by the use of different promoters, whereas the M_1 and M_2 types are produced from another single gene by alternative RNA splicing. Recently, the cDNAs for human L and M_2 type were also reported (Tani et al., 1988a,b; Kato et al., 1989). The expression of L and M-type pyruvate kinase in human was found to be tissue-specific, similar to that in rat (Tsutsumi et al., 1988).

Recently, we have shown that a cytosolic thyroid hormone binding protein (p58-M_2) present in human epidermoid carcinoma A431 cells is homologous to a subunit of pyruvate kinase subtype M_2 (PKM_2) (Kato et al., 1989). Furthermore, we also found that fructose 1,6-bisphosphate (Fru-1,6-P_2) activated the PK activity of p58-M_2 present in A431 cell lysate. We proposed that the molecular mechanism of activation was by Fru-1,6-P_2-induced tetramer formation. Because this observation was made in cellular lysate, one of the questions that arises is whether cellular cofactor(s) is (are) required for the Fru-1,6-P_2-induced association of monomeric p58-M_2 to the tetrameric PKM_2. The other question was regarding whether the monomeric p58-M_2 has intrinsic PK activity (Porter & Cardenas, 1981). To understand these problems and, furthermore, the effect of thyroid hormone on PK activity, large amounts of purified p58-M_2 are required. We, therefore, overexpressed and purified p58-M_2 to homogeneity from *Escherichia coli* and characterized its hormone binding and enzymatic activities.

Experimental Procedures

[3′-^{125}I]T_3 (2200 Ci/mmol) was obtained from Du Pont New England Nuclear. Fru-1,6-P_2, PEP, ADP, NADH/lactate dehydrogenase, and 2,4-dinitrophenylhydrazine were from Sigma. Restriction enzymes and competent *E. coli* HB101 cells were from Bethesda Research Laboratories.

Construction of the Expression Plasmid pCJ11. The expression vector (pCJ3) which contains the coding region of human placental *c-erb*A was restricted with *Nde*I and *Eco*RI.

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[1] Abbreviations: T_3, 3,3′,5-triiodo-L-thyronine; Fru-1,6-P_2, fructose 1,6-bisphosphate; PK, pyruvate kinase; DTT, dithiothreitol; T_4, L-thyroxine; Tripro, 3,3′,5-triiodothyropropionic acid; 3′-isopropyl-T_2, 3′-isopropyl-3,5-diiodo-L-thyronine; r-T_3, 3′,5′,3-triiodo-L-thyronine; PEP, phosphoenolpyruvate; ADP, adenosine diphosphate.

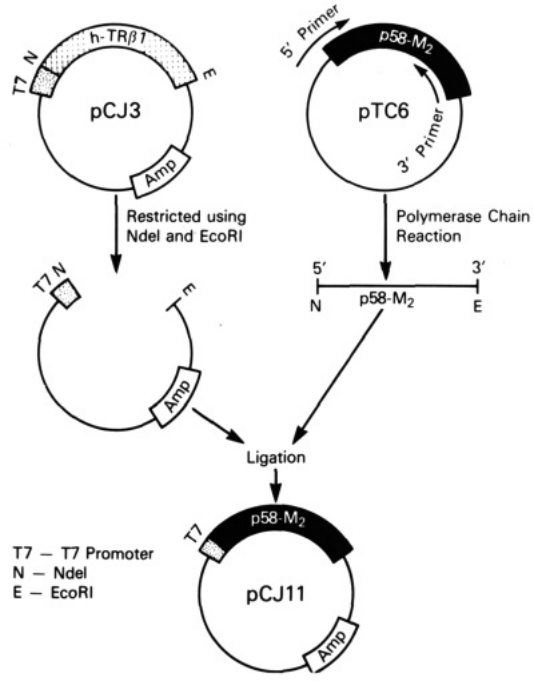

FIGURE 1: Scheme for construction of the expression plasmid pCJ11 which encodes p58-M_2. pCJ3 is the expression plasmid which encodes human placental thyroid hormone nuclear receptor (h-TRβ1) derived from pCJ2 (Lin et al., 1990). In pCJ3, a sequence of 0.455 kb from phage f1 (5488–5943) was inserted 318 bp downstream of the termination codon of h-TRβ1 in pCJ2. pTC6 contains cDNA for p58-M_2 (Kato et al., 1989).

The insert which contains the coding region of pTC6 (Kato et al., 1989) which encodes human p58-M_2 was prepared by polymerase chain reaction (PCR) using pTC6 as a template (Figure 1). Oligomers containing an *Nde*I site with the 5′ coding sequence (37-mer) or containing an *Eco*RI site together with 3′ coding sequence (42-mer) were used as primers to synthesize the insert by PCR. After purification, the insert was ligated to the vector to form pCJ11.

Expression and Purification of Human Cytosolic Thyroid Hormone Binding Protein, Subtype M_2 (p58-M_2). The expression of p58-M_2 in BL21/Lys cells was under the control of T7 promoter (Studier et al., 1990). BL21/LysS cells containing the plasmid pCJ11 were grown 37 °C in 3 L of LB broth with 100 μg/mL ampicillin and 10 μg/mL chloramphenicol to an absorbance of 0.45 at 650 nm. Isopropyl β-D-thiogalactopyranoside (1 mM) was added to induce the expression of protein for 2.5 h. All the subsequent steps were carried out at 4 °C except as stated. The cell pellet was suspended in 150 mL of buffer containing 20% sucrose, 1 mM EDTA, and 20 mM Tris, pH 7.5. After incubation for 10 min on ice, the suspension was centrifuged for 10 min at 23000*g*. The cell pellet was suspended in 150 mL of chilled water. After incubation for 10 min on ice, the suspension was centrifuged for 10 min at 7600*g*. The pellet was suspended in 30 mL of buffer P, which is phosphate-buffered saline free of Ca^{2+} and Mg^{2+} containing EDTA (5 mM) and protease inhibitors [leupeptin (1 μg/mL), phenylmethanesulfonyl fluoride (0.1 mM), and aprotinin (10 μg/mL)], and was sonicated three times (30 s each time). RNase T_1 (1.3×10^3 units) and DNase 1 (80 Kunitz units/mL) were added to the sonicated suspension and incubated at 25 °C for 30 min. One hundred twenty milliliters of buffer P was added and centrifuged for 30 min at 12000*g*. The pellet was resuspended in 120 mL of buffer W (Ca^{2+},Mg^{2+}-free phosphate-buffered saline containing 25% sucrose, 5 mM EDTA, and 1% of Triton X-100), incubated on ice for 10 min, and centrifuged for 15 min at 25000*g*. This washing step was repeated two times. The pellet was suspended in 10 mL of extraction buffer D (50 mM Tris·HCl, pH 8.0, 5 M guanidine hydrochloride, 5 mM EDTA). The suspension was sonicated for 5 s and incubated on ice for 60 min. The suspension was centrifuged at 12000*g* for 30 min. The supernatant was added to 300 mL of buffer R (50 mM Tris·HCl, pH 8.0, 1 mM dithiothreitol, 20% glycerol, protease inhibitors), which was stirred gently at 4 °C for 20 h and centrifuged at 38000*g* for 60 min. This solution contained the renatured p58-M_2.

Q-Sepharose Column Chromatography. The solution from above (330 mL) was dialyzed against buffer Q (50 mM Tris, pH 8.0, 50 mM NaCl) for 3 h at 4 °C to remove guanidine. The solution was applied to a Q-Sepharose column (2.2 × 18.4 cm) preequilibrated with buffer Q. The flow-through was collected in fractions (8 mL/fraction). Upon completion of loading the sample, the fractions from the flow-through and the washing were analyzed for T_3 binding activity and the column was further washed with 100 mL of buffer Q.

S-Sepharose Column Chromatography. The pooled fractions (340 mL) from above were dialyzed against buffer solution S (20 mM PO_4, pH 6.0, 1 mM EDTA, 0.1 mM phenylmethanesulfonyl fluoride) for 5 h at 4 °C and applied to a S-Sepharose column (2.2 × 5.3 cm) preequilibrated with buffer S. After the column was washed with 50 mL of buffer S and 30 mL of buffer S containing 50 mM NaCl, p58-M_2 was eluted with a linear gradient of pH 6.0–7.0 (200 mL) with a flow rate of 60 mL/h. The [^{125}I]T_3 binding activity was eluted at the pH of 6.15–6.3. The purity of p58-M_2 was examined by 10% SDS–polyacrylamide gel electrophoresis. The purified p58-M_2 was also evaluated by Western blotting using the two monoclonal antibodies J11 and J12 according to Obata et al. (1989).

Determination of the Molecular Size of Purified p58-M_2. The molecular size of the purified p58-M_2 was analyzed by a TSK gel (G3000 SW) column (7.5 mm × 60 cm) using a fast-pressure liquid chromatography system (FPLC; Pharmacia Co.). Purified p58-M_2 (1 mL; 0.030 mg/mL) was dialyzed against buffer F (50 mM Tris/pH 7.1, 0.1 M KCl, 5 mM $MgSO_4$) in the presence or absence of 5 mM Fru-1,6-P_2 for 3 h at 4 °C. After dialysis, the samples were loaded onto the column. The proteins were fractionated with a flow rate of 0.6 mL/min in buffer F with or without 5 mM Fru-1,6-P_2. Fractions (0.5-mL) were collected. An aliquot from each fraction (20 μL/fraction) was analyzed for pyruvate kinase activity, and an aliquot (0.2 mL/fraction) was analyzed for T_3 binding activity. The column was calibrated with bovine serum albumin (BSA) and rabbit pyruvate kinase obtained from Sigma.

Enzymatic Activity. Pyruvate kinase activities were measured by using the NADH–LDH coupled assay as described (Imamura & Tanaka, 1982), with the exception that Fru-1,6-P_2 was normally omitted from the mixture. Absorbance measurements were made in quartz cuvettes at 340 nm in a Cary 15 spectrophotometer thermostated at 25 °C. The initial rate of reaction is measured as the loss of absorbance units per minute. All reagents were from the Sigma Chemical Co. (St. Louis, MO). The specific activity in units per milligram of protein is given by

$$\text{specific activity} = \frac{\Delta A/\text{min}}{6.22(\text{concentration in mg/mL})}$$

where ΔA is the change in absorbance.

Enzymatic Kinetics. Assays were performed at varying concentrations of each substrate, PEP and ADP, while holding

the other constant, in the system described above. Three separate preparations of the protein were used in the kinetic experiments. Reported rates are the mean of two measurements at each concentration of enzyme and substrates. K_m values for each substrate, for various forms of the enzymes, were estimated by fitting values of the initial velocities and substrate concentrations to the Michaelis–Menten equation:

$$V_0 = \frac{V_{max}[ADP][PEP]}{[PEP][ADP] + K_A[PEP] + K_P[ADP] + K_PK_A}$$

where V_0 is the initial rate, V_{max} is the maximum velocity, and K_A and K_P are the Michaelis constants for ADP and PEP, respectively (Boyer, 1962). Calculations were performed by using the MLAB modeling system on the DEC-10 computer system (Knott, 1979).

Binding of $[^{125}I]T_3$ to p58-M_2. Binding of $[^{125}I]T_3$ was carried out by incubation of p58-M_2 with 0.2 nM $[^{125}I]T_3$ in the presence or absence of competitors (1–1000 nM) for 1 h at 4 °C. The protein-bound $[^{125}I]T_3$ was separated from unbound radioligand on a Sephadex G-25 (fine) column (Kitagawa et al., 1987).

Results

Expression and Purification of Cytosolic Thyroid Hormone Binding Protein (p58-M_2). Figure 1 shows the construction of the expression vector pCJ11. pCJ3 is an expression vector which encodes the human placental thyroid hormone nuclear receptor (h-TRβ1). The coding sequence of h-TRβ1 was removed from the vector and replaced with the insert which encodes p58-M_2. The expression of p58-M_2 is under the control of T7 polymerase (Studier, 1990).

Expression of p58-M_2 was induced by isopropyl β-D-thiogalactopyranoside in *E. coli*. Lane 1 of Figure 2A shows the lysate from *E. coli* which has not been transformed by the expression vector pCJ11. The protein bands were from *E. coli*. Lane 2 of Figure 2A shows that a major protein with a molecular weight of 58000 was detected in the cell lysate containing the expression vector. After lysis of the cells by osmotic shock, the two fractions, soluble and insoluble particulates, were obtained. p58-M_2 was detected in both fractions as shown in lanes 3 and 4, respectively. Comparing the amounts of p58-M_2 in lanes 3 and 4 indicated that 80–85% of the expressed protein was concentrated in the insoluble inclusion bodies. We, therefore, adopted the strategy of solubilization of p58-M_2 from the inclusion bodies using 5 M guanidine hydrochloride. p58-M_2 was allowed to renature by a 10-fold dilution of guanidine hydrochloride solution in a renaturation buffer. The renatured p58-M_2 was first purified by Q-Sepharose column chromatography. p58-M_2 was recovered in the flow-through from the Q-Sepharose column as detected by T_3 binding activity. The T_3 binding fractions were pooled, and p58-M_2 was further purified by S-Sepharose column chromatography. Elution of p58-M_2 was carried out by a linear pH gradient. p58-M_2 was eluted as a single peak at pH ~6.25. The purity of the eluted peak was established by SDS–polyacrylamide gel electrophoresis. A single protein band was seen as shown in lane 6 of Figure 2A. By use of this protocol, ~1 mg of purified p58-M_2 was obtained from 1 L of *E. coli* culture.

Previously, using the purified p58-M_2 from human epidermoid carcinoma A431 cells, we developed two monoclonal antibodies (J11 and J12) against p58-M_2 (Obata et al., 1989). Using these antibodies, we evaluated the immunoreactivity at each step of purification. Lane 2 of Figure 2B shows that one immunoreactive band with a molecular weight of 58 000 was detected in the lysate of *E. coli* transformed by pCJ11, whereas

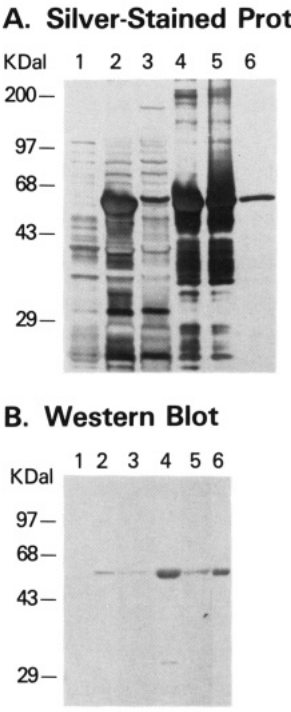

FIGURE 2: Analysis of the purity of p58-M_2 from each step of purification by silver staining (A) and Western blotting (B). (A) An aliquot of samples at each step of purification was loaded onto a 10% gel and stained with silver staining. (B) In a separate gel, the proteins were blotted onto nitrocellulose paper and reacted with monoclonal antibody J11 according to Obata et al. (1989). Lane 1, lysate (10 μg) from BL21/Lys cells; lane 2, lysate (20 μg) from BL21/Lys cells transformed by pCJ11; lane 3, soluble fraction of *E. coli* lysate (10 μg) transformed by pCJ11; lane 4, extracts from the inclusion bodies (20 μg); lane 5, Q-Sepharose flow-through (20 μg); and lane 6, the purified p58-M_2 (1.5 μg) after S-Sepharose column chromatography. The lanes in panel B are the same as in panel A except that the amounts of protein loaded onto each lane were 1/10 of those in panel A.

there is no immunoreactive band present in the control lysate (lane 1 in Figure 2B). At each step of purification, p58-M_2 reacted with J11 as shown in lanes 3–6 (Figure 2B), indicating that the purified p58-M_2 retained its immunoreactivity. Identical results were obtained with J12 (data not shown).

To ensure further that the purified protein was intact, we analyzed its amino-terminal sequence by protein sequencing. The sequence was determined to be SKPXSEAGTAF-IQTQQL. Except for the fourth amino acid (the deduced amino acid is His) which we could not identify, the amino-terminal sequence purified from *E. coli* was identical with that deduced from the cDNA sequence (Kato et al., 1989).

Purified p58-M_2 Is a Monomer and Its Association To Form Tetrameric PK Is Regulated by Fru-1,6-P_2. Figure 2 shows that the purified protein had a molecular weight of 58 000 as analyzed by a SDS-polyacrylamide gel. To determine its native molecular weight, we fractionated the purified protein on a TSK gel sizing column attached to an FPLC system. The column was calibrated by two standard markers, BSA, and commercially available rabbit PK. The eluted fractions were analyzed by both T_3 binding and PK activity. Panel A of Figure 3 shows that one T_3 binding peak was detected with a molecular size slightly smaller than that of BSA (fraction 47). However, no PK activity was detectable at the position where the standard PK was located (fraction 36). In the presence of 5 mM Fru-1,6-P_2 (panel B), T_3 binding activity was no longer detectable in fraction 47. However, a strong PK peak was detected at the position where standard PK was located. These results indicated that the p58-M_2

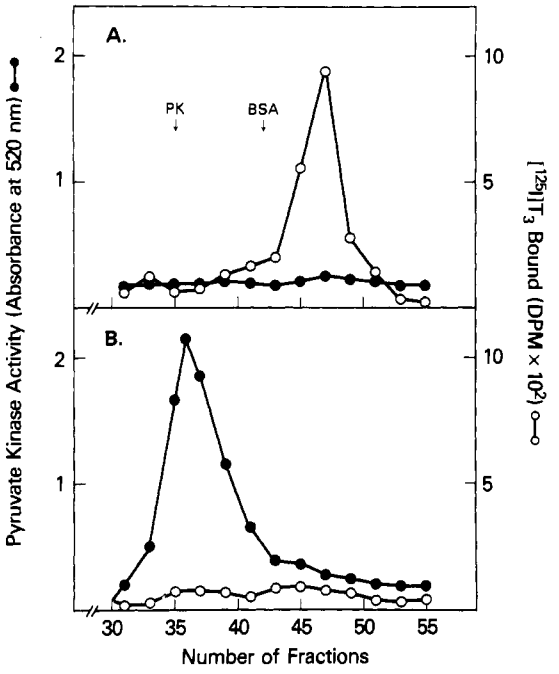

FIGURE 3: Elution profile of p58-M_2 by TSK gel column chromatography. One milliliter of purified p58-M_2 (30 μg/mL) was applied to a TSK gel column (0.75 × 60 cm) attached to an FPLC system. The protein was fractionated by using a buffer containing 50 mM Tris/pH 7.1, 100 mM KCl, and 5 mM $MgSO_4$ in the absence (panel A) or presence (panel B) of 5 mM Fru-1,6-P_2. The proteins were fractionated with a flow rate of 0.6 mL/min, and 0.25 mL/fraction was collected. Aliquots (0.2 mL) were analyzed for [^{125}I]T_3 binding (○). PK activity (20 μL/fraction) was determined (●) by the 2,4-dinitrophenylhydrazine method as described (Imamura & Tanaka, 1982).

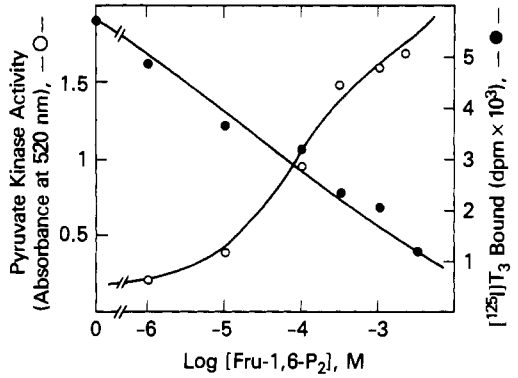

FIGURE 4: Regulation of the interconversion of p58-M_2 and PKM_2 by Fru-1,6-P_2. Purified p58-M_2 (15 μg/0.5 mL) was incubated with increasing concentrations of Fru-1,6-P_2 for 60 min at 4 °C. At the end of incubation, 5- and 200-μL aliquots were assayed for PK (○) and [^{125}I]T_3 binding activity (●), respectively.

expressed and purified from *E. coli* existed as a monomer in the absence of Fru-1,6-P_2. However, in the presence of Fru-1,6-P_2, the monomeric p58-M_2 was associated to form the tetrameric PK. Tetrameric PK did not have T_3 binding activity.

The activation of the monomeric p58-M_2 to form tetrameric PK was further evaluated by varying the concentrations of Fru-1,6-P_2. As shown in Figure 4, in the absence of Fru-1,6-P_2, p58-M_2 with high T_3 binding activity and very low kinase activity was detected (see below for the detailed description for the kinase activity of p58-M_2). Upon addition of increasing concentrations of Fru-1,6-P_2, T_3 binding activity gradually decreased, while PK activity continued to increase. At ~100 μM Fru-1,6-P_2, 50% loss of T_3 binding activity was accompanied by regeneration of ~50% of PK activity. This reciprocal relationship indicated that the formation of tetrameric PK from the monomeric p58-M_2 was regulated by Fru-1,6-P_2.

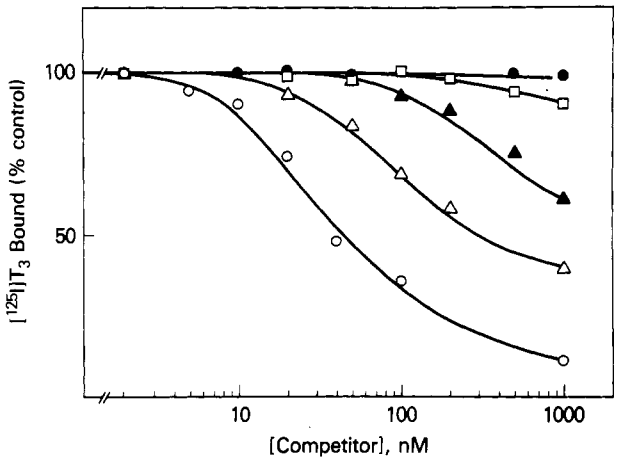

FIGURE 5: Competitive binding of T_3 and its analogues to purified p58-M_2. Purified p58-M_2 (5 μg/0.25 mL) was incubated with 0.5 nM [^{125}I]T_3 in the presence of increasing concentrations of unlabeled L-T_3 (○), L-T_4 (△), Tripro (▲), 3′-isopropyl-T_2 (□), and r-T_3 (●). The p58-M_2-bound and free [^{125}I]T_3 was separated as described under Experimental Procedures.

To evaluate the reversibility of the regulation of tetramer formation by Fru-1,6-P_2, we incubated purified p58-M_2 with 5 mM Fru-1,6-P_2 for 1 h at 4 °C to convert it to the tetrameric PKM_2. We then dialyzed the PKM_2 for 20 h at 4 °C to remove Fru-1,6-P_2. PK and T_3 binding activity were compared before and after dialysis. Ninety percent of PK activity was lost after removal of Fru-1,6-P_2 by dialysis. Concomitantly, 80–90% of T_3 binding activity was recovered. These results indicated that the regulation of interconversion of p58-M_2 and PKM_2 by Fru-1,6-P_2 is reversible.

Characteristics of the Binding of p58-M_2 to T_3 and Its Analogues. To characterize the binding of T_3 to the recombinant p58-M_2, we evaluated the effect of pH, salt, and DTT on T_3 binding to p58-M_2 purified from *E. coli* and examined its analogue binding specificity.

(*a*) *Effect of pH.* Binding of p58-M_2 to T_3 was pH sensitive. p58-M_2 lost its binding activity below pH 5. T_3 binding reached a maximum at ~pH 6.0–6.5. Further increase in pH caused the binding activity to drop. Above pH 9, no T_3 binding activity was detectable (data not shown).

(*b*) *Effect of NaCl.* NaCl had a dramatic effect on T_3 binding. T_3 binding was highest at 50 mM NaCl. Further increase in NaCl concentration reduced T_3 binding gradually. At 200 mM NaCl, 50% of T_3 activity at 50 mM NaCl was lost. When the concentration of NaCl was greater than 0.5 M, 90% of T_3 activity was lost.

(*c*) *Effects of DTT.* T_3 binding was evaluated in the presence of increasing concentrations of DTT. DTT from 0.05 to 1 mM gradually increased the T_3 binding activity. At 1 mM DTT, the binding activity was increased by 2-fold. Further increase of DTT concentration to 100 mM, however, decreased the T_3 binding to 50% of the value at 1 mM.

(*d*) *Analogy Specificity.* Figure 5 shows the results of competitive binding studies using unlabeled T_3 and four T_3 analogues. The binding constant for T_3 was calculated to be $(1.7 \pm 0.3) \times 10^7$ M^{-1} (mean ± SD, $n = 3$). Binding of p58-M_2 to T_3 analogues was also evaluated. Figure 5 shows that T_4 and Tripro competed for T_3 binding to p58-M_2. The apparent concentration of T_4 for 50% displacement was 200 nM. For Tripro, it was greater than 1 μM. 3′-Isopropyl-T_2 and r-T_3 had little or no competitive binding activity. This order of analogue competition activity is similar to p58-M_2

Table I: Kinetic Characteristics of Pyruvate Kinase Activities of p58-M_2 under Various Conditions

enzyme form	Fru-1,6-P_2	concn (ng/mL)	K_P (mM)	K_A (mM)	V_{max} (units/mg)
activated	+	50	0.13 ± 0.01	0.63 ± 0.09	490 ± 27
unactivated	-	400	2.40 ± 1.00	0.52 ± 0.26	104 ± 26
unactivated	-	150	1.55 ± 0.73	3.85 ± 2.40	22 ± 9

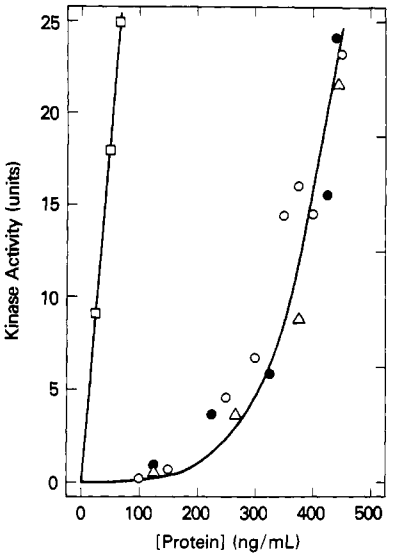

FIGURE 6: Dependence of the rate of pyruvate kinase activity on p58-M_2 concentration. The rate of enzymatic activity was determined in the standard assay conditions (2 mM PEP and ADP). p58-M_2 (10–50 μg/mL) was preincubated with 2 mM Fru-1,6-P_2 (□), 2 mM alanine (△), or no preincubation (○). The activity was determined after a 100-fold dilution of the preincubation mixture with the assay buffer. Activity of p58-M_2 was also determined in the presence of 0.5 mM Fru-1,6-P_2 in the assay buffer with no preincubation (●). The line through the data is drawn by assuming a concentration-dependent equilibrium between an inactive monomer and a tetramer of specific activity 250 units/mg. The value of the association constant used is 0.04 nM^{-3}. These values were derived by using the equation 4p58-M_2 = PKM_2 and by fitting the data using the MLAB (Knott et al., 1979) modeling system.

identified in A431 cells (Kitagawa et al., 1987). However, this order of analogue competition for binding activity is very different from that reported for thyroid hormone nuclear receptor, in which 3′-isopropyl-T_2 binds more strongly than T_3 (Bolger & Jorgensen, 1980).

The Purified p58-M_2 Has Intrinsic PK Activity. The availability of p58-M_2 in purified form enabled us to characterize its kinetic properties. By use of the NADH–LDH coupled assay, the enzymatic activity in the absence or presence of Fru-1,6-P_2 was studied. Following activation by preincubation with 2 mM Fru-1,6-P_2, p58-M_2 showed a high specific activity of 370 units/mg determined under the assay conditions described by Imamura and Tanaka (1982). The initial rate was a linear function of enzyme concentration over the range investigated (0–500 ng/mL). The unactivated enzyme showed more complex behavior. At low concentrations, the enzyme showed very low activity. However, the measured rates increased in a nonlinear way with increasing protein concentration (Figure 6). This concentration-dependent increase in specific activity was not prevented by preincubation of the p58 monomer with a low concentration of L-alanine (20 μM), or by inclusion of 0.5 mM Fru-1,6-P_2 in the assay mixture.

The enzymatic activity of p58-M_2 was measured as a function of substrate concentrations. Under all circumstances, within experimental error, its behavior could be fitted to the Michaelis–Menten equation, indicating the absence of cooperative interactions seen in some other forms of the enzyme (Eigenbrodt et al., 1983). Panel I of Figure 7 shows the

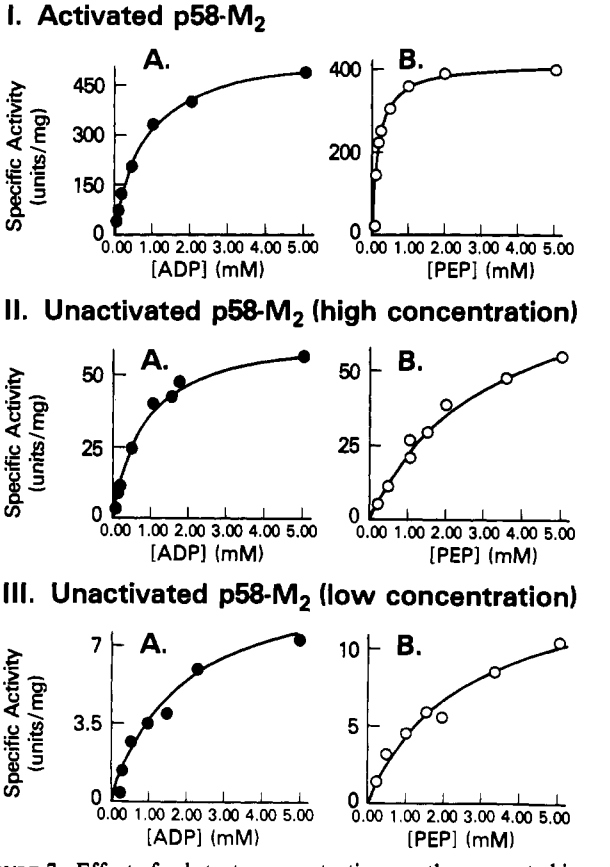

FIGURE 7: Effect of substrate concentrations on the pyruvate kinase activity of p58-M_2. (I) Activity was determined after preincubation of p58-M_2 with 2 mM Fru-1,6-P_2 at 4 °C. (A) Variation of ADP concentration, with 2 mM PEP; (B) variation of PEP concentration, with 2 mM ADP. Protein concentration was 50 ng/mL. (II) Effect of substrate concentrations on the pyruvate kinase activity of unactivated p58. (A) Variation of ADP concentration, with 2 mM PEP; (B) variation of PEP concentration, with 2 mM ADP. Protein concentration was 400 ng/mL. (III) Effect of substrate concentrations on the pyruvate kinase activity of unactivated p58. (A) Variation of ADP concentration, with 2 mM PEP; (B) variation of PEP concentration, with 2 mM ADP. Protein concentration was 150 ng/mL.

dependence of the pyruvate kinase activity of p58-M_2 on PEP and ADP concentrations, following activation by preincubation in 2 mM Fru-1,6-P_2. Under this condition, p58-M_2 was associated to form PKM_2 (see Figure 3). Fitting all the data to the Michaelis–Menten equation gave the values listed in Table I. The K_m for ADP (K_A) and K_m for PEP (K_P) of PKM_2 derived after activation of p58-M_2 in the presence of Fru-1,6-P_2 are 0.63 ± 0.10 mM and 0.13 ± 0.01 mM, respectively. The specific activity (V_{max}) at saturation concentrations of both substrates (PEP and ADP) was 490 ± 27 units/mg. The V_{max} and K_A were comparable to PKM_2 obtained from AH-130 cells (Imamura & Tanaka, 1982). However, the K_P for the present recombinant PKM_2 was one-third of that of the PKM_2 isolated from AH-130 cells (Imamura & Tanaka, 1982).

The behavior of the unactivated enzyme was more complex (panels II and III of Figure 7). When assayed at a high protein concentration (400 ng/mL), the values of K_A and K_P were 0.52 ± 0.2 mM and 2.40 ± 1.0 mM, respectively. The apparent maximum activity of the enzyme was 104 ± 26

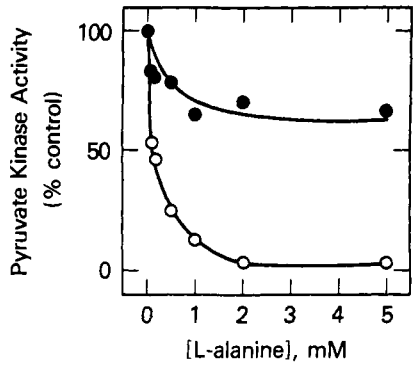

FIGURE 8: Inhibition of pyruvate kinase activity of p58-M_2 by L-alanine. The activity of p58-M_2 was determined under standard assay conditions (2 mM PEP and 2 mM ADP) with (●) or without (○) preincubation with 2 mM Fru-1,6-P_2. The final alanine concentrations are shown.

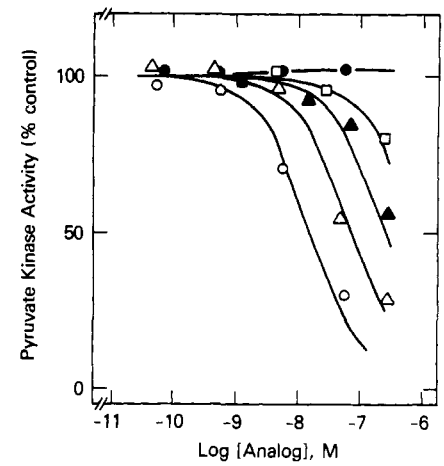

FIGURE 9: Dose-dependent inhibition of pyruvate kinase activity of p58-M_2 by the thyroid hormones. Purified p58-M_2 was incubated with increasing concentrations of T_3 (○), T_4 (△), Tripro (▲), 3′-isopropyl-T_2 (□), and r-T_3 (●) for 2 h at 4 °C. An aliquot (100 ng/0.25 mL) was assayed under standard conditions (2 mM PEP, 2 mM ADP).

units/mg. At a lower protein concentration, 150 ng/mL, values of K_A and K_P were 3.85 ± 2.40 mM and 1.55 ± 0.73 mM, respectively. The apparent maximum specific activity of the enzyme at this concentration was 22 ± 9 units/mg. Activation by Fru-1,6-P_2 also altered the enzyme's apparent inhibition by L-alanine (Figure 8). Under standard assay conditions, the presence of 2 mM alanine reduced the activity of unactivated p58 by 97%. After preincubation in 2 mM Fru-1,6-P_2, the same concentration of inhibitor produced only 30% inhibition.

Inhibition of the Kinase Activity of p58-M_2 by Thyroid Hormone. p58-M_2 binds T_3 and exhibits analogue specificity. We evaluated the effect of hormone binding on the enzymatic activity of p58-M_2. Figure 9 shows that the kinase activity of p58-M_2 was inhibited by T_3, T_4, Tripro, and 3′-isopropyl-T_2 in a concentration-dependent manner. In contrast, the enzymatic activity of p58-M_2 was not affected by r-T_3. Analysis of the inhibition curves yielded the apparent K_I's of 20 nM, 100 nM, 300 nM, and 1 μM for T_3, T_4, Tripro, and 3′-isopropyl-T_2, respectively. The extent of inhibition for these analogues was parallel with their binding activity (see Figure 5). The inhibitory effect of thyroid hormone, however, is reversible. The inclusion of 2 mM Fru-1,6-P_2 led to the reversal of this inhibitory effect. Under such circumstances, as illustrated in Figure 4, tetrameric PKM_2 was formed and PK activity was activated.

Discussion

In the present study, we overexpressed monomeric p58-M_2 and purified it to apparent homogeneity. Analysis by amino-terminal sequencing, molecular size determination, immunoreactivity with two monoclonal antibodies, and its ability to form active tetrameric PK indicated that purified p58-M_2 retains its native structure. Comparison of the T_3 binding characteristic under different pH, salt, and DTT concentrations indicated that p58-M_2 isolated from *E. coli* and isolated from human A431 cells (Kitagawa et al., 1987) are virtually indistinguishable. Previously, we reported the yield of purified p58-M_2 obtained from A431 cells was ~50 μg from 2.5 × 10^9 cells. Approximately 1 mg of purified p58-M_2 was obtained from 1 L of culture. Therefore, the present study provided a convenient method to obtain a large quantity of the pure monomeric form of PKM_2 which was unobtainable before.

Previously, purification of p58-M_2 from A431 cells necessitated the use of the detergent 3-[(3-cholamidopropyl)dimethylammonio]-1-propanesulfonic acid, which was not possible to be removed subsequently from the purified p58 (Kitagawa et al., 1987). The purification of large amounts of detergent-free p58-M_2 described in the present study enabled us to characterize its enzymatic properties and thyroid hormone binding activity in its native state. The availability of the purified p58-M_2 in the native state led us to resolve the issue of whether the monomer of PK possesses intrinsic kinase activity (Porter & Cardenas, 1981). Measurement of pyruvate kinase activity over a much wider range of protein concentrations than was possible in earlier experiments indicates a nonlinear dependence of activity on protein concentration (Figure 6). However, when the protein was activated by preincubation with Fru-1,6-P_2, its enzymatic activity showed the usual linear dependence on concentration. These observations are most easily explained by a model which assumed that, under these assay conditions, p58-M_2 is in a concentration-dependent equilibrium between monomer and tetramer. The curve drawn in Figure 6 indicates that this nonlinear behavior can be approximated by increased formation of a tetramer of high specific activity at higher concentrations of p58-M_2.

The results shown in Figure 7 and Table I allow us to compare the kinetic properties of p58-M2 with those previously reported for pyruvate kinase enzymes from other sources (Imamura & Tanaka, 1982; Kayne, 1973). In general, most nonmuscle forms of the enzyme show a significant degree of positive cooperativity in the binding of PEP, but not ADP. Activation by Fru-1,6-P_2 results from a change of the PEP saturation curve to a noncooperative hyperbolic form, with a lowering of the apparent value of K_P. Within experimental error, our data fail to reveal any cooperativity in the response of p58-M_2 to PEP or ADP. However, the derived values of the K_m's for both substrates are in the millimolar concentration range usually observed for PKM_2 forms of the enzyme. Activation by Fru-1,6-P_2 produces an apparent 20-fold decrease in the K_m for PEP. As might be expected, the kinetic behavior of the unactivated enzyme is a function of protein concentration. Within experimental error, the K_m for PEP is similar at both 400 and 150 ng of protein/mL, whereas the K_m for ADP is increased 7-fold at the lower concentration (Table I). According to our model, in the absence of Fru-1,6-P_2, the protein exists as a mixture at all examined concentrations: the predicted compositions are 70% monomer and 30% tetramer at a concentration of 150 ng/mL and 30% monomer and 70% tetramer at 400 ng/mL. Thus, none of these constants are characteristics of a pure species, but they can be taken as

evidence that we are observing the behavior of different forms of the enzyme: mainly an unactivated tetramer at high concentrations and a monomer of very low specific activity at low concentrations of protein. Conversion of monomer to tetramer results in an increase in specific activity and a decrease in K_A. In agreement with previous observations, activation of the tetramer by Fru-1,6-P_2 was accompanied by a decrease in K_P. The pyruvate kinase activity of p58-M_2 was strongly inhibited by millimolar concentrations of L-alanine, and this inhibition was partially relieved by preincubation with Fru-1,6-P_2 (Figure 8). So far, we have been unable to demonstrate formation of tetrameric p58-M2 by FPLC measurements in the absence of Fru-1,6-P_2, suggesting that the formation of tetramer is linked to the binding of PEP and/or ADP.

On the basis of these results, it is reasonable to propose that the enzyme exists in a dynamic equilibrium between a monomer of low specific activity as a kinase and a tetramer with high specific activity. The equilibrium constant for this association seems to depend on buffer conditions. T_3 and its analogues bind to the monomeric p58-M_2, inhibiting its enzymatic activity and preventing association to a tetramer. In contrast, Fru-1,6-P_2 binds strongly to the tetrameric form of the protein, increasing its specific activity and favoring formation of the tetramer. Furthermore, on the basis of the observation that 50% of conversion of monomer to tetramer occurred at a Fru-1,6-P_2 concentration of $\sim$100 μM in an A431 cell lysate described earlier (Kato et al., 1989) and purified p58-M_2 described in the present study, it would seem that other cellular factors are not required for such regulation.

The existence of two interconvertible forms of PKM_2 was reported earlier by Imamura et al. (1972) and others (Bailey et al., 1968; Susor & Rutter, 1968; Pogson, 1968a,b; Flanders, 1971). Imamura et al. observed a Fru-1,6-P_2-sensitive and a Fru-1,6-P_2-insensitivie form of PKM_2 (Imamura et al., 1972). In the absence of Fru-1,6-P_2, PEP concentration-dependent enzymatic activity exhibited a biphasic form. After incubation of the enzyme preparation with 0.1 mM of Fru-1,6-P_2 at 37 °C for 10 min, the PEP saturation curve was transformed to a monophasic, normal Michaelis–Menten type identical with the present results shown in Figure 7 for the tetrameric PK. The authors had determined the sedimentation coefficients for Fru-1,6-P_2-sensitive and -insensitive form to be 9.71 and 9.44 S, respectively. However, the molecular mechanism for these interconversions was unknown. Our present results suggested that these two interconvertible forms described by Imamura et al. could correspond to the monomeric p58-M_2 and the tetrameric PKM_2. The Fru-1,6-P_2-dependent conversion of the two forms could be explained by the perturbation of the monomer–tetramer equilibrium.

The novel regulating mechanism observed in vitro in the present study may not be limited to PKM_2. Several laboratories have also reported an apparent interconversion of Fru-1,6-P_2-sensitive and -insensitive forms of PKs from rat liver (L type) (Bailey et al., 1968; Susor & Rutter, 1968), rat epididymal adipose tissue (Pogson, 1968a,b), and heart muscle of *Rana pipiens* (M_1 type) (Flanders et al., 1971). It would not be surprising that the monomer–tetramer interconversion could also be regulated by thyroid hormone in a Fru-1,6-P_2-dependent way since PKM_2 shares high sequence homology to the three other subtypes of PK. However, the verification of this hypothesis will have to await the results of future studies.

Acknowledgments

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Registry No. ADP, 58-64-0; PEP, 73-89-2; Fru-1,6-P_2, 488-69-7; T_3, 6893-02-3; T_4, 51-48-9; Tripro, 51-26-3; 3'-isopropyl-T_2, 51-23-0; r-T_3, 5817-39-0; PK, 9001-59-6.

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