Turnover of Hepatic Phosphofructokinase in Normal and Diabetic Rats

ROLE OF INSULIN AND PEPTIDE STABILIZING FACTOR*

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Earlier work demonstrated that the activity of liver phosphofructokinase (PFK-L2) and immunoactive PFK-L2 were decreased in diabetic rats and increased to normal or super-normal amounts following insulin treatment (Dunaway, G. A., and Weber, G., (1974) Arch. Biochem. Biophys. 162, 629-637). This report indicates that the decrease in levels of PFK-L2 in diabetic rats is a result of an accelerated degradation rate while the synthetic rate remains nearly normal. Following insulin treatment, the rate of PFK-L2 synthesis is enhanced 2-fold, whereas the rate of degradation appears to be greatly diminished. An inverse relationship is shown to exist between the PFK-L2 levels and the rates of PFK-L2 degradation, suggesting that the levels of PFK-L2 are primarily regulated by degradation rate. In addition, the levels of the PFK-L2 peptide stabilizing factor are inversely proportional to rates of PFK-L2 degradation. These results indicate that insulin mediates the rate of degradation of PFK-L2 by controlling the level of the peptide stabilizing factor.

The utilization of carbohydrate in energy production and glycogen deposition as well as its entry into protein and lipid metabolism is under elaborate and precise control. Insulin plays an important role in this complex regulatory process (1). It is well known that hepatic glycolysis and gluconeogenesis is sensitive to blood levels of insulin (1, 2). The flow of carbohydrate is thought to be largely a consequence of the state of flux at each of the unidirectional enzymes of glycolysis and gluconeogenesis (2). Through the efforts of many investigators, it is known that one of the important actions of insulin is to regulate hepatic carbohydrate metabolism by affecting the activities of the key gluconeogenic enzymes, glucose-6-phosphatase (2, 3), fructose-1,6-bisphosphatase (2), pyruvate carboxylase (4), and phosphoenolpyruvate carboxykinase (4, 5), as well as the key glycolytic enzymes, glucokinase (6), phosphofructokinase (7), and pyruvate kinase (2, 8). For example, diminished insulin levels as in diabetes mellitus result in a decrease in the ratio of the activities of glucokinase/glucose-6-phosphatase, phosphofructokinase/fructose-1,6-bisphosphatase, and pyruvate kinase/phosphoenolpyruvate carboxylase. This imposes an abnormally large gluconeogenic flux which contributes to the hyperglycemic state during diabetes.

The role of insulin in regulation of one of the key glycolytic enzymes, phosphofructokinase, has been extensively examined (2, 7, 9, 10). It has been shown that the activity of hepatic phosphofructokinase was decreased in diabetic rats (2, 7). Its activity could be increased following insulin treatment but this increase was blocked by actinomycin-D (2). Subsequent work demonstrated that only the major liver phosphofructokinase isozyme was insulin sensitive while the minor PFK1 isozyme exhibited little response to insulin (7).

In this report it is indicated that in diabetic rats the decrease in the levels of PFK-L2 is a consequence of an enhanced degradation rate while maintaining a normal synthetic rate. The increase in PFK-L2 activity following insulin treatment appears to be a consequence of an enhanced rate of synthesis as well as a significantly diminished degradation rate. A recently discovered regulatory molecule (10) appears to mediate insulin-induced alterations of PFK-L2 content. The results presented here suggest that the PFK-L2 peptide stabilizing factor (9, 10) mediates insulin action by affecting the susceptibility of PFK-L2 to intercellular protein degradation.

EXPERIMENTAL PROCEDURES

Rats of the Wistar strain weighing 180 to 200 g were caged in a Stay-Clean laminar flow system (Lab Products, Inc., Garfield, N. Y.) and illuminated daily from 7:00 a.m. to 7:00 p.m. Purina laboratory rat chow and tap water were available ad libitum. Following decapitation and exsanguination, livers were rapidly removed, and the lungs were examined for pneumonitis. Only rats visibly free of pneumonitis were used in the diabetes experiments. PFK-L2 and the stabilizing factor were measured as previously described (9, 10). The induction of diabetes with alloxan and the maintenance of diabetic rats with insulin have been described elsewhere (7). PFK-L2 was purified with the following modifications of the method of Dunaway and Weber (7). An initial pH of 8.5 of the extraction buffer was used in order to attain a pH of 8.0 following homogenization. In subsequent steps a pH of 8.0 was maintained as described in the original method. At Step 3 the ammonium sulfate concentration was increased to 35% saturation (205 mg/ml). At Step 4 DEAE-Sepharose CL-6B was substituted for DEAE-cellulose and the method of elution remained unchanged. Further, the fractions of highest activity were concentrated by nitrogen pressure filtration using the Amicon YM-10 membrane. This new membrane from Amicon Corp. allows almost complete enzyme recovery in the final concentrate. At Step 6 the fractional precipitation was as before with the exception that the heat denaturation step was eliminated. The final step after fractional precipitation was chromatography on Sepharose CL-4B and elution with 50 mM Tris (pH 8.0), 50 mM ammonium sulfate, 1.0 mM ATP, and 10 mM dithiothreitol. The enzyme elutes immediately after the void volume and appears to be homogeneous on 1% agar electrophoresis and SDS-polyacrylamide gel electrophoresis.

Previously, we had reported its subunit molecular weight to be 65,000 by disc PAGE. By the techniques of slab PAGE (see last paragraph of this section for methodology), we find the subunit molecular weight to be 80,000 to 85,000. This difference appears to be

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1 The abbreviations used are: PFK, phosphofructokinase; PFK-L2, the major liver phosphofructokinase isozyme; SDS, sodium dodecyl sulfate; PAGE, polyacrylamide gel electrophoresis.

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a consequence of the different destaining techniques used in the two techniques. The disc gels were vertically destained in the electrophoresis cell, whereas the slab gel was destained by diffusion with repeated changes of the destaining solution. Since vertical electrophoretic destaining could have caused further migration of the subunit, resulting in a decrease in the estimation of its molecular weight, we repeated the earlier work with the disc gels using either diffusion or horizontal electrophoretic destaining techniques. These data confirm that PFK-L1 has a subunit molecular weight of 80,000 to 85,000.

Antiserum was produced by an initial injection of 6 mg of enzyme/rabbit in Freund’s complete adjuvant. Significant antibody titers were found after 3 weeks at which time each rabbit received a booster injection of 6 mg of enzyme with Freund’s incomplete adjuvant. After 10 days, maximum titers of antienzyme antibody were found. Each rabbit was bled, and the antiserum was processed as previously described (7). Immunodiffusion studies with this antiserum against PFK-L2 as well as liver supernatants from normal, diabetic, and insulin-treated diabetic rats produces a single line of identity, which indicates its effectiveness in recognizing PFK-L2 in normal as well as diabetic and insulin-treated diabetic animals.

The measurement of incorporation of [1-3,4,5-3H]leucine into the enzyme has been described (7). The samples were counted in 10 ml of Ready-Solv HP (Beckman Instruments, Inc.). All counting was performed in Beckman LS-3130 liquid scintillation counter. Quenching was determined using the automatic external standard.

The rate of degradation of the enzyme was determined using the [14C]carbonate incorporation method (11). Each rat was injected (intraperitoneally) with 0.5 mCi of NaH14CO3 (New England Nuclear) in phosphate-buffered saline. At appropriate intervals five rats were killed, the enzyme was immunoprecipitated, as described in the legend of Fig. 1, and the amount of label remaining was measured as described in the previous paragraph.

The purity of SDS/urea-treated immunoprecipitates from rat liver supernatant fluids was determined by SDS-PAGE. Antibody precipitates were obtained from rats which had been injected with 1 mCi of NaH14CO3 as described in the previous paragraph, as described in legend of Fig. 1, the dissociated immunoprecipitates as well as similarly treated PFK-L0 and IgG were subjected to SDS-PAGE using the Ortec 4200 vertical slab gel electrophoresis system and 4100 pulsed constant power supply.

RESULTS

Purity of PFK-L2-Antibody Precipitates—As shown in the stained gel in Fig. 1, SDS/urea treatment of the immunoprecipitate produces two detectable proteins. The slower appears to be IgG and the faster corresponds to the subunit of PFK-L2. In addition, most (about 80%) of the radioactivity is located in region of the PFK-L2 subunit, indicating the effectiveness of the antibody and methodology in isolating PFK-L2 from rat liver supernatant fluids.

Rates of Synthesis of PFK-L2—The rate of synthesis of PFK-L2 was determined by measuring the rate of incorporation of [3H]leucine into immunopreipitate enzyme (Table I). The apparent rate of synthesis in the normal and diabetic rat were identical. Insulin treatment of the diabetic rats caused a 92 and 69% increase in the relative rates of synthesis after 24 and 48 h, respectively.

| Condition          | [3H]Leucine incorporation into PFK-L2 (dpm × 10^3) | Phosphofructokinase (dpm/g) | Phosphofructokinase/supernatant protein (×10^3) | Relative rate of synthesis |
|--------------------|--------------------------------------------------|-----------------------------|-----------------------------------------------|---------------------------|
| Normal             | 206 ± 29                                         | 1146 ± 101                  | 5.56 ± 1.49                                  | 1.00                      |
| Diabetic            | 187 ± 24                                         | 1013 ± 84                   | 5.42 ± 1.31                                  | 0.97                      |
| Insulin treatment   |                                                  |                              |                                               |                           |
| 1 day              | 216 ± 34                                         | 2309 ± 148                  | 10.69 ± 2.81                                 | 1.92                      |
| Insulin treatment   |                                                  |                              |                                               |                           |
| 2 days             | 236 ± 30                                         | 2219 ± 195                  | 9.40 ± 2.32                                  | 1.69                      |
Degradation Rate Constant for PFK-L2—The steady state degradation rate constant \( k_d \) for PFK-L2 was determined using a single pulse of \([^{14}C]\)carbonate (11). A steady state half-life of 1.8 days was determined for PFK-L2 from data in Fig. 2. From this half-life a \( k_d \) of 0.38 (day)\(^{-1}\) was calculated using the first order rate equation, \( k_d = 0.693/t_{1/2} \). Using double-labeling methodology (\([^{14}C]\)- and \([^{3}H]\)leucine), we previously reported a \( k_d \) of 0.1 (day)\(^{-1}\) (7). Since measurement of hepatic turnover rates with \([^{14}C]\)carbonate yields faster and seemingly better degradation rate constants (11), this difference is likely a result of the methodology which was employed. As shown in Fig. 3, the half-life for PFK-L2 is significantly decreased in diabetic rats (0.6 day) as compared to the normal rat (1.8 days). No measurable levels of radioactive PFK-L2 were detectable in the diabetic rat 4 days post injection.

Table II

| Condition                  | PFK Activity | \( \Delta \)PFK/\( \Delta t \) | \( \Delta \)Syn/\( \Delta t \) | \( \Delta \)Deg/\( \Delta t \) |
|----------------------------|--------------|-------------------------------|-------------------------------|-------------------------------|
| Normal                     | 2.50         | 0.96\(^{a}\)                  | 0.96\(^{a}\)                  | 0.96\(^{a}\)                  |
| Diabetic                   | 0.60         | -0.95\(^{a}\)                | 0.96\(^{a}\)                  | -1.91\(^{a}\)                |
| Insulin-treated (1 day)    | 2.30         | 1.70                         | 1.84                         | 0.14                         |
| Insulin-treated (2 days)   | 3.15         | 0.85                         | 1.62                         | 0.77                         |

\(^{a}\)Expressed in units of phosphofructokinase activity per mg of DNA per day.

\(^{b}\)The steady state rates of synthesis and degradation were calculated assuming steady state conditions (\( \Delta \)Syn/\( \Delta t \)) = (\( \Delta \)Deg/\( \Delta t \), assuming a first order of degradation, i.e. \( k_d \) [PFCL2], and using the \( k_d \) for PFK-L2 (0.38 day\(^{-1}\)) from the data in Fig. 2.

\(^{c}\)The change in amount of phosphofructokinase was calculated by subtraction of the previous level and division by number of elapsed days.

\(^{d}\)The non-steady state rates of synthesis were calculated by multiplying the normal steady state rate of synthesis by the relative rate of phosphofructokinase synthesis from the last column of Table I.

\(^{e}\)The non-steady rates of degradation were calculated from the equation (\( \Delta \)Deg/\( \Delta t \)) = (\( \Delta \)Syn/\( \Delta t \)) - (\( \Delta \)PFK/\( \Delta t \)).

\(^{f}\)The insulin treatment was as described in Fig. 1.

The effects of diabetes and insulin treatment on the rates of synthesis and degradation of PFK-L2 are shown in Table II. The steady state rates of synthesis and degradation were calculated by multiplication of the steady state \( k_d \) (Fig. 2) times the steady state level of PFK-L2. In the diabetic liver the rate of synthesis of PFK-L2 appeared to be at a normal rate, although the level of PFK-L2 decreased 75% during this period. Thus, the loss in amount of PFK-L2 appeared to be at a normal rate, although the level of PFK-L2 decreased 75% during this period. The loss in amount of PFK-L2 can be explained by an increased rate of enzyme degradation. The decreased half-life of PFK-L2 in diabetic rat liver (see Fig. 3) confirms this conclusion. Following insulin treatment, the level of PFK-L2 increases from a low level in diabetic liver to near normal after 24 h, and above normal in 48 h. Insulin treatment of diabetic rats causes the
rate of synthesis of PFK-L₂ to be increased 84% after 24 h and increased 62% after 48 h of insulin therapy. After 24 h, the apparent rate of degradation of PFK-L₁ is markedly reduced but increases to near normal rates in the next 24-h period.

Variations of the PFK-L₁ and PFK-L₂ Peptide Stabilizing Factor Levels—In the diabetic rat liver, the stabilizing factor is decreased 90% while PFK-L₂ is decreased 75% (Fig. 4). Following the administration of insulin, the amount of factor increases 30-fold, becoming maximal after 8 h and remaining as such for approximately 16 h. The levels of PFK-L₂ increase at the greatest rate during this 24-h period. The factor decreased to near normal levels after 48 h of insulin treatment, and PFK-L₁ levels increased to 125% above normal. Earlier studies covering only the first 24 h agree quite well with the reported data (10).

**DISCUSSION**

Other work has indicated that the hepatic PFK-L₁ activity decreased during diabetes, while after insulin treatment it increased to normal in 24 h and to above normal after 48 h (7). This report extends the earlier work by demonstrating that the loss of PFK-L₁ during diabetes is a consequence of enhanced degradation and correlates with loss of factor. Further, the increased PFK-L₁ levels after the first 24 h of insulin treatment are a consequence of both an enhanced rate of synthesis and a severely depressed rate of degradation and are characterized by an approximate 30-fold increase in factor compared to diabetic levels. During the next 24 h the rate of degradation of PFK-L₁ and amount of factor returned to near normal, but the rate of synthesis remained elevated.

Since the rate of PFK-L₁ synthesis in the untreated diabetic rat remains normal, it is apparent that a consistent correlation does not exist between the rate of PFK-L₁ synthesis and serum insulin amounts, stabilizing factor levels, or changes in PFK-L₁ concentration. However, the rates of PFK-L₁ degradation were inversely related to serum insulin levels, amount of stabilizing factor, and changes in PFK-L₁ concentration. The relationship between the rates of PFK-L₁ degradation and PFK-L₂ or stabilizing factor levels can be seen by considering the data in Table II and Fig. 4. In diabetic rat liver the level of PFK-L₁ and the level of factor were decreased while the rate of degradation was markedly enhanced. Following insulin treatment, a significant increase in levels of the factor preceded any measurable change in PFK-L₁. Also, during this 24-h period the rate of degradation appeared to be strongly inhibited and the levels of PFK-L₁ increased. During the period from 24 to 48 h following initiation of insulin treatment, the level of factor and rate of degradation of PFK-L₁ returned to normal. Further, increases in the level of PFK-L₂ appeared to lessen. These data strongly implicate the factor as a mediator of insulin action via modulation of PFK-L₁ degradation rates. That this factor is involved in a general mediation of insulin action via modulation of PFK-L₁ degradation rates. That this factor is involved in a general mediation of insulin action via modulation of PFK-L₁ degradation rates. That this factor is involved in a general mediation of insulin action via modulation of PFK-L₁ degradation rates.

Another possible aspect of regulation of liver PFK has been reported regarding its interconvertibility between active (phosphorylated) and inactive (dephosphorylated) forms (13, 14). Although confirmation of these findings has not appeared elsewhere, isolation of muscle PFK in a phosphorylated state has been reported (15). It has been suggested that the amount of PFK protein remains constant in fasted livers, although the PFK activity decreases significantly, and it was concluded that only the proportion of active PFK is reduced (13, 14). We have reported that the reduction of activity in fasted rats is accompanied by a decrease in immunoreactive PFK (7). A possible explanation of this difference is a decreased immunoreactivity of the inactive PFK.

Recently, we have attempted to determine the presence of inactive PFK in diabetic livers. Incubation of diabetic rat liver supernatant fluids which were prepared by our methods (9), with 6 mM ATP for 15 min at 25°C, the PFK activation conditions described by Brand and Soling (13) and Brand et al. (14), produced no change in total PFK activity. However, after 1 h of incubation, a 0 to 10% increase in activity was observed. These data indicate that little if any inactive PFK is present in diabetic rat liver supernatants as prepared by our procedures (9). Thus, the possibility that two forms of PFK exist under our conditions seems remote, and the potential problem of a decreased precipitation of inactive PFK seems unlikely. Following immunoprecipitation of PFK activity from supernatant fluids of diabetic rat liver, a measurable increase in the unprecipitated PFK activity (about 15% of the total) using the previously described PFK activation conditions could not be detected. Seemingly, this confirms that inactive PFK is not present following immunoprecipitation with anti-PFK-L₁ antiserum.

While the interconversion of PFK may play an important role in an immediate response to nutritional or hormonal changes, it appears from our results that the decrease in PFK-L₁ activity is maintained during chronic fasting or diabetes, which are strong protein catabolic states, by loss of PFK-L₁ protein through enhanced degradation of PFK-L₁ caused by decreased levels of the peptide stabilizing factor. Further, it is suggested that the recovery of PFK-L₁ following insulin therapy is largely a result of decreased degradation which is a consequence of supernormal levels of factor. Finally, it is plausible to propose that the turnover of the factor is regulated by insulin and that it mediates the action of insulin by controlling the susceptibility of PFK-L₁ to degradation.

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