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RAPID INACTIVATION OF RAT LIVER PHOSPHO*ENOL*PYRUVATE CARBOXYKINASE BY MICROSOMES AND REVERSAL BY REDUCTANTS *

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Summary

The hepatic gluconeogenic enzyme phospho*enol*pyruvate carboxykinase (GTP:oxaloacetate carboxy-lyase (transphosphorylating), EC 4.1.1.32) is activated 3—4-fold by micromolar quantities of Fe^{2+} and ferroactivator, a cytosol protein that is necessary for this activation. After the carboxykinase in liver cytosol, or the purified carboxykinase, was activated by adding Fe^{2+}, rat liver microsomes were added to duplicate their ratio to the carboxykinase in liver. Microsomes caused a biphasic inactivation of the carboxykinase in cytosol. All of the activity corresponding to Fe^{2+} activation was lost within 10 min ($t_{1/2}$ equaled 3—4 min) and the remaining (basal) activity was lost over hours ($t_{1/2}$ equaled 3—4 h). Adding the reducing agent dithiothreitol (1 mM) after 10 or 60 min of exposure of cytosol to microsomes caused a complete and three-fourths complete restoration of Fe^{2+} activation. In preventing the microsome-induced loss of Fe^{2+} activation, ascorbate and cysteine were also effective. When the purified carboxykinase was activated by Fe^{2+} and the synthetic ferroactivator 3-aminopicolinate, microsomes interfered with the enzyme activity in a manner identical to their effect on the enzyme in cytosol, suggesting that they did not act on ferroactivator protein. When Fe^{2+} was added to the crude or the purified carboxykinase 5—60 min after microsomes, the carboxykinase was not activated. If the microsomes were acting by oxidizing or sequestering Fe^{2+}, the carboxykinase would have been at least transiently

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Abbreviation: Zwittergent 3-14, *N*-tetradecyl-*N*,*N*-dimethyl-3-ammonio-1-propanesulfonate; Hepes, *N*-2-hydroxyethylpiperazine-*N'*-2-ethanesulfonic acid.

activated because it takes only seconds for Fe^{2+} to fully activate the enzyme and minutes for microsomes to cause a complete loss of Fe^{2+} activation. By the process of elimination, microsomes probably act on the carboxykinase itself. Microsomes did not inactivate five other glycolytic/gluconeogenic enzymes in liver cytosol, including some that have sulfhydryls which are very sensitive to oxidation. The microsomal factor was inactivated by the proteases, chymotrypsin, trypsin and pronase. The activity of the microsomal factor was highest in liver and kidney; almost as high in heart as in liver; and low or negligible in pancreas, spleen, lung and skeletal muscle. The microsomal activity was decreased in livers of alloxan diabetic rats, and the decrease was proportional to their blood glucose concentrations — such that severely diabetic rats had almost none of the activity. Liver microsomes from rats treated with the antioxidant, vitamin E, were completely without effect on Fe^{2+} activation. The results indicate that it is possible to alter rapidly the Fe^{2+} activation of phospho*enol*-pyruvate carboxykinase by mechanisms that probably involve oxidation/reduction. The decreases in the microsomal factor induced by alloxan diabetes; its high content in the gluconeogenic organs, liver and kidney; and its apparent specificity for the carboxykinase are preliminary evidence of the microsomal factor having a physiological role in the regulation of the carboxykinase.

Lardy and coworkers [1—9] have hypothesized for a variety of reasons that activation of the gluconeogenic enzyme phospho*enol*pyruvate carboxykinase by micromolar quantities of Fe^{2+} is a means of regulation of the enzyme and of gluconeogenesis. One of the modes of regulation might involve controlling the amount of Fe^{2+} available to the enzyme and in support of this hypothesis they have found a protein in liver named ferroactivator protein that is necessary for Fe^{2+} to activate the carboxykinase [4,5]. Another mode of regulating the activity of the enzyme in vivo might be by altering its ability to be activated by Fe^{2+}. We now report that there is a factor in liver microsomes that interferes with the ability of phospho*enol*pyruvate carboxykinase to undergo activation by Fe^{2+}. The properties of the microsomal factor and the response of the carboxykinase to the microsomes we studied bear some resemblances to those described by Ballard and Hopgood [10] who found that microsomes irreversibly inactivated the carboxykinase with a $t_{1/2}$ of 3—4 h, but that reducing agents prevented the inactivation. Although they did not study the effect of microsomes on activation of the enzyme by micromolar amounts of Fe^{2+}, they did test whether millimolar quantities of Fe^{2+} protected the enzyme against inactivation by microsomes (it did not) *. We found that the loss of Fe^{2+} activation of the carboxykinase induced by microsomes was rapid with a $t_{1/2}$ of about 4 min and that reducing agents not only prevented, but reversed, the loss of Fe^{2+} activation of the enzyme.

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* Millimolar quantities of several transition metal ions, including Fe^{2+}, can by themselves inactivate the carboxykinase.

Materials and Methods

Animals. Sprague-Dawley rats weighing 200—250 g were starved for 24 h prior to death unless mentioned otherwise. Some rats were made diabetic with alloxan, 37 mg/kg body weight, intravenously, 10—14 days before death.

Materials. Auxiliary enzymes used in the assays were from Boehringer Mannheim, Indianapolis, IN, U.S.A. The sources of all other materials were mentioned previously [6,7]. If not, they were from P-L Biochemicals, Milwaukee, WI, U.S.A., Boehringer Mannheim, or Sigma Chemical Co., St. Louis, MO, U.S.A., in the highest purity available. Phospho*enol*pyruvate carboxykinase was purified from rat liver cytosol according to the procedures of Ballard and Hanson [11] and Philippidis et al. [12] incorporating the modifications of Columbo et al. [13]. The specific activity of the purified enzyme when assayed at 25°C in the absence of effectors and in the direction of phospho*enol*-pyruvate formation was 14 μmol/min per mg protein (expressed as 100% enzyme activity in Fig. 3).

Preparation of subcellular fractions. Livers from rats starved for 24 h were perfused with 154 mM NaCl to remove blood and were used to prepare cytosol and microsomes. All buffers were degassed with reduced pressure and then gassed with nitrogen to remove oxygen. Cytosol was the supernatant fraction obtained from centrifuging a 1 : 3 (w/v) homogenate of liver in 10 mM triethanolamine chloride, pH 7.5, and 0.25 M sucrose at 105 000 × *g* for 1 h. The activity of phospho*enol*pyruvate carboxykinase in the cytosol was 3.3—4.7 μmol/min per g liver when assayed at 25°C in the direction of phospho*enol*-pyruvate formation and without prior incubation with effectors (expressed as 100% enzyme activity in Figs. 1, 2 and 4—6).

Microsomes were prepared by a conventional method [14] using 10 mM triethanolamine chloride, pH 7.5, and 0.25 M sucrose for all steps. The original homogenate of liver was centrifuged at 600 × *g* for 10 min to sediment the nuclear fraction and the supernatant fraction centrifuged at 15 000 × *g* for 10 min to obtain the mitochondrial fraction. The resulting supernatant fraction was centrifuged at 105 000 × *g* for 1 h to sediment the microsomal fraction. The microsomal fraction was centrifuged at 15 000 and 105 000 × *g* once more to wash the microsomes and then they were resuspended in a volume of the buffered sucrose equivalent to 0.8 the weight of the liver and stored frozen at −20°C or used immediately. The activity of the microsomal factor remained constant in samples stored for up to 1 month. When microsomal fractions were prepared from liver and the other organs to compare their relative abilities to interfere with the Fe^{2+} activation of phospho*enol*pyruvate carboxykinase the organs were not perfused with NaCl because this could alter the weights of the livers. The nuclear and mitochondrial fractions were washed twice by centrifuging for 10 min at 600 × *g* and 15 000 × *g*, respectively.

Enzyme assays. All solutions were degassed with reduced pressure for at least 30 min and gassed with nitrogen or argon for at least 30 min prior to use to remove oxygen, although omitting these steps did not alter the results in any way.

Phospho*enol*pyruvate carboxykinase activity was assayed in the direction of phospho*enol*pyruvate formation as previously described [4,7,9,15]. Prior to

assaying for the activity cytosol (which contains the carboxykinase and ferroactivator protein), or the purified enzyme, was incubated at 37° C in 5 mM Hepes buffer, pH 7.2, with or without $FeCl_2$ and effectors. After 10 min microsomes were added to duplicate their ratio to cytosol (or to phospho*enol*pyruvate carboxykinase activity when purified enzyme was used) in intact liver. The amounts of cytosol and microsomes in 1 ml of incubation mixture were equal to those in 20—30 mg liver except when stated otherwise. 0.1-ml aliquots were withdrawn from the incubation mixture at intervals and added to 0.9 ml enzyme reaction mixture to assay for phospho*enol*pyruvate carboxykinase activity at 25° C.

The activity of other enzymes in liver cytosol was assayed by the methods cited: fructose bisphosphatase [16], glyceraldehyde phosphate dehydrogenase [17], phosphoglycerate kinase [18] phosphofructokinase [19] and pyruvate kinase [20].

Results

Effect of microsomes on phosphoenolpyruvate carboxykinase in cytosol

Liver cytosol contains ferroactivator protein necessary for the Fe^{2+} activation of phospho*enol*pyruvate carboxykinase. 10 min after phospho*enol*pyruvate carboxykinase in cytosol was activated by Fe^{2+} (activation is complete within 5 s [7]), adding an amount of microsomes to cytosol that duplicated their ratio in whole liver caused a biphasic loss of phospho*enol*pyruvate carboxykinase activity (Fig. 1). All of the carboxykinase activity

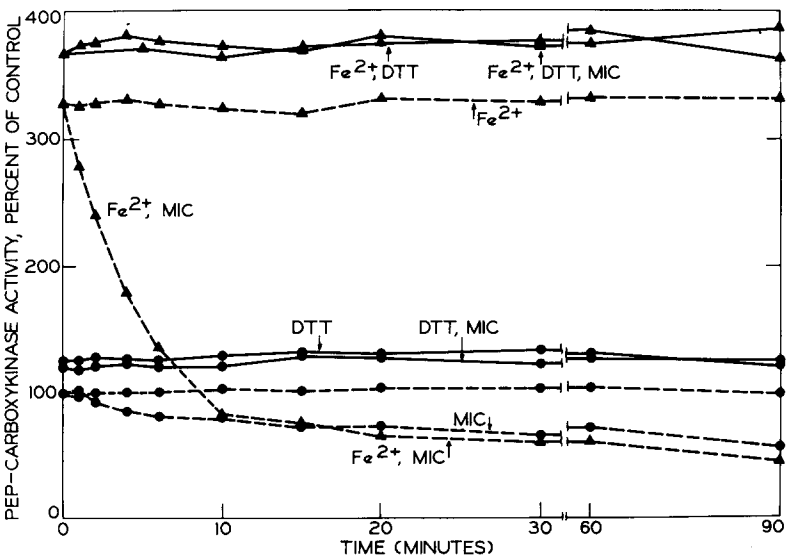

Fig. 1. Effect of microsomes on the activity of phospho*enol*pyruvate carboxykinase. Liver cytosol was incubated with 30 μM $FeCl_2$ (Fe^{2+} and triangles), or without it (circles), and with 1 mM dithiolthreitol (DTT and solid lines), or without it (dashed lines). After 10 min (at time zero in the figure) microsomes (MIC) or additional buffer (unlabeled) were added, and at intervals, aliquots were withdrawn to assay for enzyme activity. The activity equal to 100% is the activity at time zero in cytosol incubated without any effectors. The amounts of cytosol and microsomes in 1 ml of incubation mixture were equal to those from 25 mg (wet weight) intact liver.

corresponding to Fe^{2+} activation was lost within 10 min ($t_{1/2}$ = 3—4 min) of adding the microsomes and the remaining activity was lost more slowly ($t_{1/2}$ = 3—4 h). After 90 min of exposure to microsomes an activity remained that corresponded to 43% of the control i.e., the activity of cytosol that had not been reacted with added Fe^{2+} or any other effector before assaying for enzyme activity. Adding the reducing agent dithiothreitol (1 mM) to the incubation mixture before microsomes were added completely prevented both the first- and second-phase losses of enzyme activity. Incubating cytosol with microsomes, but without added Fe^{2+}, caused a slight but rapid loss of carboxykinase activity indicating that Fe^{2+} endogenous to the cytosol was probably causing a slight activation of the phospho*enol*pyruvate carboxykinase.

Fig. 2 shows that adding 1 mM dithiothreitol to the cytosol after 10 or 60 min of exposure to microsomes caused a complete and three-fourths complete restoration of Fe^{2+} activation of phospho*enol*pyruvate carboxykinase, respectively. The fact that the first-phase loss of activity was completely reversible indicates that the effect of the microsomes on the Fe^{2+} activation of the carboxykinase does not involve primarily a proteolytic degradation of the enzyme and suggests the possibility of a mechanism involving oxidation and reduction for rapid control of the enzyme. However, since all of the carboxykinase activity could not be restored by the dithiothreitol after 1 h exposure to micro-

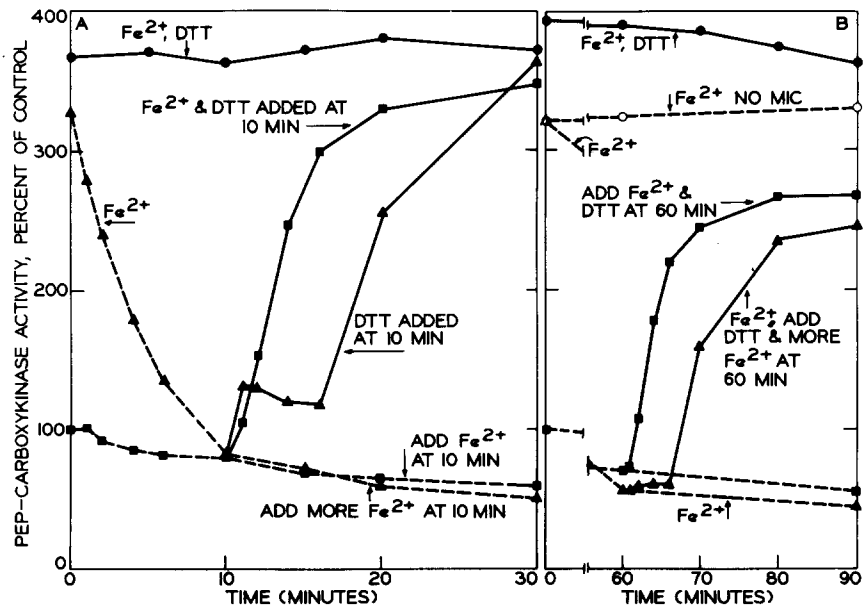

Fig. 2. Effect of adding dithiothreitol on the activity of phospho*enol*pyruvate carboxykinase after exposing it to microsomes. Liver cytosol was incubated for 10 min with or without 30 μM $FeCl_2$ (Fe^{2+}) before microsomes were added at time zero. Microsomes were added to all of the mixtures except the control indicated by open circles. 10 min (A) or 60 min (B) after the microsomes were added, 1 mM dithiothreitol (DTT) alone or with Fe^{2+}, or Fe^{2+} alone, were added. The triangles and sqaures indicate the mixture to which Fe^{2+} was and was not added initially, respectively. A control to which microsomes were added at time zero, (10 min after Fe^{2+} and dithiothreitol) is indicated by closed circles. Solid and dashed lines indicated when a mixture did and did not contain dithiothreitol, respectively. Other conditions are as described in the legend to Fig. 1.

somes, the second-phase loss of activity could involve proteolysis. (If the percentage of Fe^{2+} activation had been calculated on the basis of the activity at 60 min of the cytosol incubated without Fe^{2+} but with microsomes, then the percentage of Fe^{2+} activation would have been equal to that of the enzyme not exposed to microsomes, indicating that the slow phase loss of activity might be due to a loss of the catalytic activity per se, of the enzyme.) Adding Fe^{2+} to cytosol after 10 min of exposure to microsomes did not cause any activation of the enzyme unless dithiothreitol was added with the Fe^{2+}. Because added Fe^{2+} was not present in the incubation mixture during the period of inactivation, the microsomes could not have caused a loss of enzyme responsiveness to the Fe^{2+} by oxidizing or sequestering Fe^{2+}. Also consistent with this idea is that adding more Fe^{2+} to the cytosol, to which Fe^{2+} and microsomes had been previously added, did not even partially increase the activity of the enzyme. Because activation of the fresh enzyme by Fe^{2+} is complete within 5 s and is thus more rapid than inactivation by microsomes [7] (Fig. 2), if the microsomes had acted directly on Fe^{2+}, at least a transient increase in enzyme activity should have occurred upon addition of more Fe^{2+}.

Effect of microsomes on purified phosphoenolpyruvate carboxykinase

To test whether the microsomes act on the carboxykinase itself, or other factors in liver cytosol such as ferroactivator protein, 3-aminopicolinate, a synthetic ferroactivator [7,9] was used to permit Fe^{2+} to activate the purified carboxykinase. Fig. 3A shows that microsomes caused a rapid loss of Fe^{2+} activation of the purified enzyme and that the Fe^{2+} activation was restored by adding 1 mM dithiothreitol. When the carboxykinase was first exposed to microsomes for 5 or 20 min and then 3-aminopicolinate and Fe^{2+} were added, the enzyme was not activated unless dithiothreitol was added simultaneously with the Fe^{2+} (Fig. 3B) indicating that, as with the enzyme in crude cytosol, Fe^{2+} does not need to be present during the inactivation of the enzyme. Since ferroactivator protein was not present in the experiments shown in Fig. 3 and 3-aminopicolinate does not contain any groups that are readily oxidized, the results indicate that microsomes act directly on the carboxykinase. Also, the fact that dithiothreitol not only prevents, but reverses the inactivation of the enzyme, indicates that dithiothreitol is acting directly on the carboxykinase and not merely inhibiting the microsomal factor, although it was not proved that it is not doing the latter in addition to reacting with the carboxykinase.

Characteristics of the microsomal factor and carboxykinase inactivation

It was determined that the loss of Fe^{2+} activation of phospho*enol*pyruvate carboxykinase in cytosol induced by microsomes is a first-order reaction by plotting the log of the rate of carboxykinase inactivation vs. time. By plotting the $t_{1/2}$ values of carboxykinase Fe^{2+} activation in the presence of various concentrations of liver microsomes a quantitative assay for the factor that inactivates the carboxykinase was developed. With this assay it was determined that greater than 97% of the total activity in liver that interfered with the Fe^{2+} activation of phospho*enol*pyruvate carboxykinase was contained in the microsomal fraction. The rest of the activity was in the mitochondrial fraction and the nuclear and cytosolic fractions contained almost no activity.

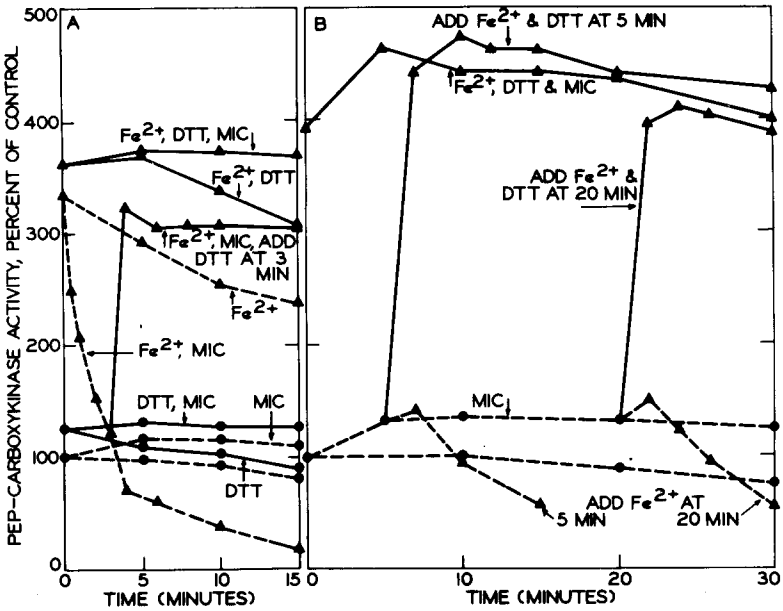

Fig. 3. Effect of microsomes, dithiothreitol and Fe^{2+} and 3-aminopicolinate on the activity of purified phospho*enol*pyruvate carboxykinase. Purified carboxykinase was incubated for 10 min with or without 1 mM dithiothreitol (DTT) and/or 200 μM $FeCl_2$ and 1 mM 3-aminopicolinate before microsomes (MIC) or additional buffer (unlabeled) at time zero. 3-aminopicolinate was added whenever $FeCl_2$ was added (Fe^{2+}). When not added initially, dithiothreitol alone, or with Fe^{2+}, was added at the times shown. Triangles and solid lines indicate when Fe^{2+} and dithiothreitol, respectively were present. Other conditions are as described in the legend of Fig. 1.

The microsomal factor that interferes with the Fe^{2+} activation of the carboxykinase appears to have a protein component as it was inactivated by incubating microsomes with the proteases, chymotrypsin, trypsin and pronase when incubated 14 h at 22°C in the proportion of 1 part of protease to 50 parts microsomes (n = four experiments). The factor was also inactivated by 2.4% perchloric acid, precipitated by 40% $(NH_4)_2SO_4$ and solubilized by several detergents including 0.5% Triton X-100, 0.5% lubrol and 1.5% Zwittergent 3-14.

However, it was stable to boiling for 10 min. Solubilization of the factor with Triton X-100 or Zwittergent 3-14 and chromatography (in various orders) on Sephadex G-100, or Biogel A 1.5 m and CM-cellulose and DEAE-cellulose did not separate the factor from the bulk of the microsomal protein whether or not the detergents were included in the chromatography buffers. These findings are similar to those of Ballard and Hopgood [10] and support their suggestion that the factor(s) is (are) tightly associated with the microsomal membranes. Such an association could prevent a heat-induced change in structure that would cause inactivation of a protein.

Effect of reductants on inactivation

The loss of Fe^{2+} activation of phospho*enol*pyruvate carboxykinase induced by microsomes was interefered with by several reducing agents besides dithio-

threitol. Under the conditions used in the experiments shown in Figs. 1 and 2 in which the ratio of microsomes to cytosol was equal to their ratio in liver, only ascorbate (1 mM), besides dithiothreitol, prevented and reversed the microsome-induced loss of Fe^{2+} activation. Cysteine (1 and 3 mM) and reduced glutathione (1 and 3 mM) were without effect (data not shown). When the ratio of microsomes to cytosol was adjusted so that their ratio was one-fifth that in intact liver, then 1 mM cysteine protected against the inactivation of the carboxykinase. But reduced glutathione 1 mM β-mercaptoethanol and 1 mM sodium dithionite did not significantly protect the enzyme against inactivation.

Heavy metal ions contained in the microsomal fraction could produce an inactivation of phospho*enol*pyruvate carboxykinase by oxidizing sulfhydryls on the enzyme and such an inactivation might, of course, be reversed by reducing agents. In addition to its reducing effect, the protecting effect of dithiothreitol could involve chelation of metals. It would appear unlikely that the contents of metals in the microsomal fractions of rat liver are high enough to cause an inactivation of the carboxykinase [21,22]. The contents of many transition metals in rat liver are lower than 1 μmol/kg liver [21] and the microsomes were diluted 1 : 40 or more when they were incubated with cytosol or the pure carboxykinase. Dialyzing 2 ml microsomes (1 : 8 dilution) in two 1 l changes of 20 mM EDTA in 20 mM Hepes buffer, pH 7.5, for 48 h to remove metals did not alter the microsomal activity. Ashing the microsomes which should destroy all but metals resulted in a complete loss of the microsomal activity.

Lipid peroxides readily form in liver microsomal membranes when liver is homogenized and the microsomal fraction is isolated and then stored [23—25]. Peroxides formed from the autooxidation of unsaturated fatty acids in the microsomal membrane might inactivate phospho*enol*pyruvate carboxykinase by oxidizing sensitive groups on the enzyme and such an effect might be prevented by reducing agents. Inconsistent with this mechanism is the constancy of the microsomal activity after repeated freezings and thawings of the microsomal fraction and during incubation at 37°C for 90 min and the amounts in liver of well-fed rats or rats starved 24 h always varied within a narrow range. Preparing microsomes in buffers that were degassed with reduced pressurefor 30 min and then gassed with nitrogen and/or argon for 30 min to remove oxygen and then adding dithiothreitol, or EDTA, both of which should decrease the formation of lipid peroxides [23,26,27], did not alter the microsomal activity that interferes with the Fe^{2+} activation of the carboxykinase. NADPH cytochrome *c* reductase of liver microsomes can mediate lipid peroxidation. Adding NADPH or NADH to mixtures of pure or impure carboxykinase and microsomes did not alter the rate of inactuvation of the carboxykinase.

To test more specifically whether lipid peroxides could interfere with the Fe^{2+} activation of phospho*enol*pyruvate carboxykinase, arachidonic methyl ester was incubated with liver cytosol at 37°C under various conditions that induce the production of lipid peroxides, such as with Fe^{2+} and ascorbic acid [26—28]. The methyl arachidonate produced a slight loss of the Fe^{2+} activation of the carboxykinase as might be expected. However, the fastest rate of inactivation was only one-fourth as fast as that seen with microsomes diluted

such that the ratio of microsomes to cytosol was one-fifth that in liver, and the amount of lipid peroxides formed by the methyl arachidonate were 10^3—10^4 greater than in the microsomal membranes whether the water soluble peroxides were estimated by malondialdehyde formation [26—29] or the water insoluble peroxides were measured by an iodometric assay after extraction with chloroform/methanol [29]. Thus, the extent of the loss of Fe^{2+} activation of the carboxykinase induced by microsomes did not correlate at all with the amount of lipid peroxidation.

Incubating the microsomes with an equal weight of catalase for 30 min before and during their exposure to the carboxykinase did not at all interfere with the inactivation of the carboxykinase. This is against the idea that the microsomes inactivate the carboxykinase by producing H_2O_2.

Administering sodium phenobarbital (75 mg/kg intraperitonally), a well known inducer of microsomal oxidases [23], to rats for 3 days prior to killing did not alter the microsomal activity.

Effect of tocopherol on the microsomal factor

Rats were given α-tocopherol acetate, 50 mg/kg body weight, intraperitoneally 12 h prior to killing. The liver microsomes isolated from these rats were completely ineffective in interfering with the Fe^{2+} activation of phospho*enol*-pyruvate carboxykinase (Fig. 4). One of the known effects of tocopherol is inhibition of lipid peroxidation, but in view of the results of the previous sec-

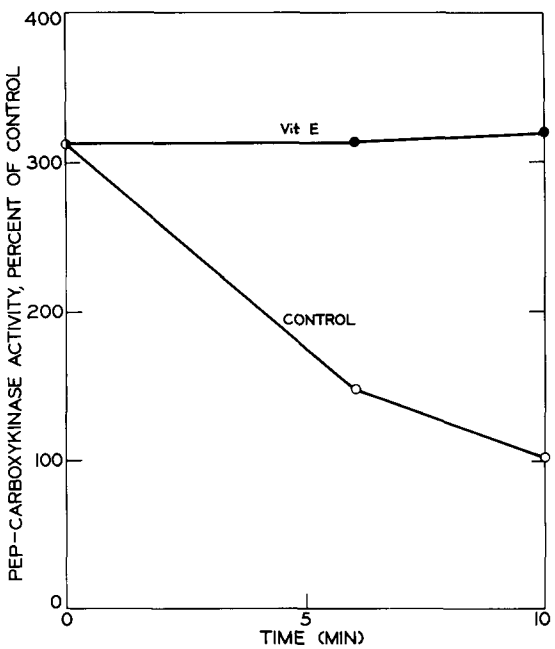

Fig. 4. Effect of vitamin E on the microsomal activity that interferes with Fe^{2+} activation of phospho*enol*pyruvate carboxykinase. Rats (n = 8) were treated with α-tocopherol acetate (50 mg/kg) 12 h before microsomes were isolated. Liver cytosol was incubated with 30 μM $FeCl_2$ for 10 min before microsomes equivalent to those from 5 mg liver were added at time zero to make the final volume 1 ml. There were four control rats. Other conditions were the same as described in the legend to Fig. 1.

tion, the effect of tocopherol must involve another of its anti-oxidant functions.

Effect on other enzymes

The ability of microsomes to inactivate other glycolytic/gluconeogenic enzymes in rat liver cytosol was tested to find out whether microsomes could inactivate other enzymes containing sulfhydryl groups and whether this effect of microsomes was at all specific for phospho*enol*pyruvate carboxykinase. When microsomes were incubated with liver cytosol for 20 min, they did not alter the activities of the following enzymes even when their activities were assayed in the presence of known effectors of some of the enzymes: fructose bisphosphatase, phosphofructokinase, (in the presence of 1 mM AMP) glyceraldehyde phosphate dehydrogenase, phosphoglycerate kinase and pyruvate kinase (in the presence or absence of 75 μM fructose bisphosphate). Some of these enzymes, especially glyceraldehyde phosphate dehydrogenase [30], but also fructose bisphosphatase [16] and phosphofructokinase [19,31] are known to have sulfhydryls that are very sensitive to oxidation with a concomitant loss of enzyme activity.

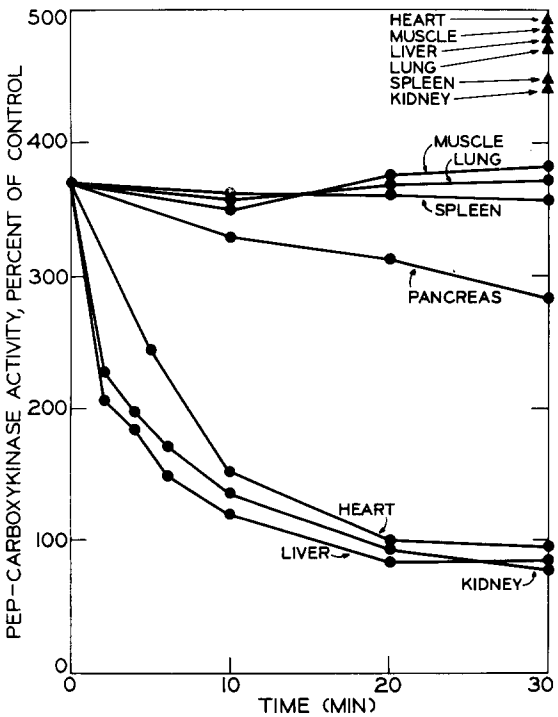

Fig. 5. Effect of microsomes from various rat organs on the Fe^{2+} activation of phospho*enol*pyruvate carboxykinase. Liver cytosol was incubated with 30 μM $FeCl_2$ for 10 min before a microsomal fraction equivalent to that from 12.5 mg (wet weight) of the intact organ was added at time zero (circles) to make the final volume 1 ml. The triangles indicate the effect of adding dithiothreitol (1 mM) at the beginning of the incubation period. Other conditions were the same as described in the legend to Fig. 1.

Tissue distribution

Microsomes from liver and kidney contained the highest amounts of activity that interferes with the Fe^{2+} activation of phospho*enol*pyruvate carboxykinase. Microsomes from heart contained nearly as much activity as liver and kidney, pancreas much less activity and microsomes from skeletal muscle, lung and spleen contained essentially no activity. The activity in all of the tissues was eliminated by dithiothreitol (Fig. 5).

Effect of alloxan diabetes on the microsomal factor

Normal rats, either starved 24 h or well fed, had the highest content of the factor in liver microsomes that interferes with the Fe^{2+} activation of phospho-*enol*pyruvate carboxykinase.

Fig. 6 shows the effects of adding microsomes isolated from alloxan diabetic rats with various degress of hyperglycemia to liver cytosol pooled from normal rats and then measuring the activity of phospho*enol*pyruvate carboxykinase. Because the activity of the carboxykinase is markedly increased in alloxan

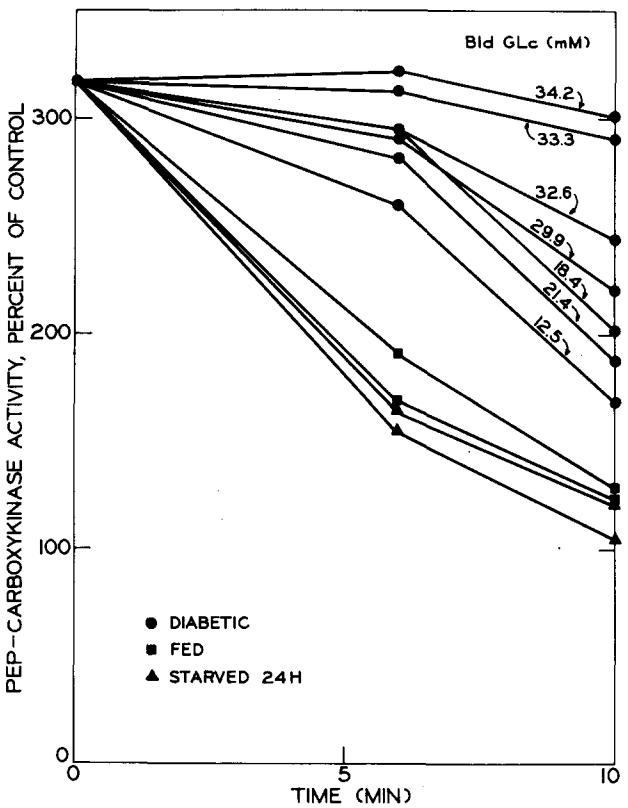

Fig. 6. Effect of alloxan diabetes on the microsomal activity that interferes with the Fe^{2+} activation of phospho*enol*pyruvate carboxykinase. Liver cytosol was incubated with 30 μM $FeCl_2$ for 10 min before microsomes equivalent to those from 5 mg intact liver were added at zero time to make the final volume 1 ml. The blood-glucose values of the diabetic rats are shown near each curve. The effects of microsomes from two rats starved 24 h and two well-fed rats are also shown. Other conditions were as described in the legend to Fig. 1.

diabetes [6,32] and gluconeogenesis is accelerated in diabetes, a decrease in a factor that inactivates the enzymes might be expected. The ability to interfere with the Fe^{2+} activation of the enzyme was inversely correlated with the hyperglycemia and microsomes from the more severely diabetic rats had almost no activity — thus suggesting that the activity of the microsomal factor might be under hormonal control. The protein contents of the microsomes from diabetic rats (mean ± S.D., 20.0 ± 0.7, $n = 8$) were not very different from those of starved rats (23.2 ± 1.1, $n = 12$) or fed rats (23.7 ± 1.4 mg/g liver, $n = 6$).

Discussion

Previous work showed that the activity of phospho*enol*pyruvate carboxykinase in vitro is increased 3—4-fold by micromolar amounts of Fe^{2+} [3—7,9]. The results of the present study demonstrate that the activity of the enzyme in vitro can also be controlled by altering its ability to respond to Fe^{2+}. Incubating liver microsomes with the crude enzyme in cytosol, or the purified enzyme, before assaying for enzyme activity rapidly interferes in a time-dependent manner ($t_{1/2} = 3$—4 min) with the ability of th enzyme to be activated by Fe^{2+}; and this effect is prevented and rapidly reversed by some reducing agents. The ability of the enzyme to undergo subsequent activation by Fe^{2+} is lost when it is treated with microsomes before Fe^{2+} is added. The untreated enzyme can be activated by Fe^{2+} in seconds [7] and the microsome-induced unresponsiveness to Fe^{2+} requires minutes so it is unlikely that the action of the microsomes involves oxidation or sequestration of Fe^{2+} because if it did, at least a transient increase in activity of the enzyme should have occurred when Fe^{2+} was added.

Since microsomes interfere with the Fe^{2+} activation of the enzyme in a system containing only the pure enzyme, Fe^{2+} and a synthetic ferroactivator 3-aminopicolinate, a small molecule that does not contain any groups that are sensitive to oxidation, it is likely that the microsomes act on the carboxykinase itself.

The effect of the microsomes, to the extent tested, is specific for phospho*enol*pyruvate carboxykinase. The activities of other enzymes in liver cytosol, including glyceraldehyde phosphate dehydrogenase, phosphofructokinase [19,30] and fructose bisphosphatase [16] that contain sulfhydryls that are sensitive to oxidation with a concomitant loss of enzyme activity, were not affected by the microsomes. Liver and kidney, the two organs that contain the highest contents of phospho*enol*pyruvate carboxykinase and have the highest capacities to synthesize glucose, contain high amounts of the microsomal factor. However, heart, which contains little of the carboxykinase, has almost as much of the microsomal factor as liver and kidney. Pancreas contains a small amount of the microsomal activity and skeletal muscle, lung and spleen contain none of this activity. In livers of alloxan diabetic rats the content of the microsomal factor is inversely correlated with the blood glucose levels of the rats. Since the carboxykinase is increased 5—7-fold in alloxan diabetes in the rats [6,31], a decrease in a factor that inactivates the carboxykinase might be expected and is consistent with hormonal effects on the microsomal factor.

In addition to causing the rapid loss of Fe^{2+} activation of the carboxykinase,

microsomes cause a slow loss of the catalytic activity of the carboxykinase. Our findings of a half-life of 2—3 h for the latter process and that the slow inactivation is prevented, but not reversed, by some reducing agents confirm those of Ballard and Hopgood [11] who described a microsomal factor that causes a slow loss of catalytic activity of the carboxykinase. Whether their factor is the same factor that causes the rapid loss of Fe^{2+} activation of the enzyme has yet to be determined. Many of the characteristics of the two activities besides their intracellular location are similar, such as their sensitivity to proteolytic enzymes and the difficulty encountered in isolating the factors from the bulk of the microsomal protein even with detergents, [11] indicating that each of them could be intimately associated with the microsomal membranes.

Since the effect of microsomes is rapidly reversed by some reducing agents and phospho*enol*pyruvate carboxykinase contains three sets of vicinal dithiols [22], an attractive hypothesis for the mechanism of action of the microsomes on the enyzme involves the oxidation of the dithiols to intramolecular disulfides. Experiments to prove that the microsomes act by such a mechanism cannot be done until the microsomal factor is purified to remove proteins that might interfere with analyzing the sulfhydryl content of the enzyme. Nevertheless, it is tempting the speculate that the physiologic mechanisms involved in regulating the carboxykinase could involve oxidation/reduction. There are some connections between redox changes and the regulation of metabolism, and even to reactions that could be related to gluconeogenesis in mammals. It has been well established that in plants, the light dependent transfer of electrons from chlorphyll to ferredoxin and then to thioredoxin via ferredoxin-thioredoxin reductase can activate at least six enzymes important to plant metabolism [33]. Less thoroughly delineated are interactions between hormones and redox changes in mammalian systems. Czech and coworkers [34] have shown the an effect of insulin at a step subsequent to interaction with its receptor in the fat cell involves the oxidation of sulfhydryls and Morgan et al. [35] have suggested that sulfhydryls are involved in insulin action in the rat diaphragm. Löw and Crane and coworkers [36,37] have shown that physiologic concentrations of insulin and glucagon can inhibit and activate, respectively, NADH oxidoreductases in both the plasma membrane and the endoplasmic reticulum from mouse liver. Kulinski and Kolesnichenko [38] have shown that cyclic AMP stimulates a protein kinase in mouse liver that activates a disulfide reductase. Because glucagon probably acts via cyclic AMP, it could potentially stimulate disulfide reduction in liver. At this time, these associations are coincidental. However, the redox changes, i.e., oxidation by insulin and reduction by glucagon, are in the directions expected if insulin, which inhibits, and glucagon, which activates gluconeogenesis, were to control gluconeogenesis in part by acting on phospho*enol*pyruvate carboxykinase.

To our knowledge, the microsomal activity described in the present report is the only factor, naturally-occurring or synthetic, that interferes primarily with the Fe^{2+} activation of phospho*enol*pyruvate carboxykinase without simultaneously interfering with the catalytic activity per se of the enzyme. Although the decrease in the microsomal factor induced by alloxan diabetes, its high content in the gluconeogenic organs, liver and kidney, and its apparent specificity for the carboxykinase are compatible with it being a physiologic

regulator of the carboxykinase much more conclusive evidence for this supposition is needed.

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