*ACTIVATION OF RABBIT LIVER FRUCTOSE DIPHOSPHATASE BY COENZYME A AND ACYL CARRIER PROTEIN**

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Abstract.—The catalytic activity of rabbit liver fructose diphosphatase is enhanced more than fourfold by treatment with coenzyme A or acyl carrier protein. Other sulfhydryl compounds, such as glutathione or cysteine, are without effect. Activation is reversed by reduced glutathione or cysteine, indicating that the enzyme and activator are linked by disulfide bridges. The activated enzyme derivative can be formed by a disulfide exchange reaction at alkaline pH or by an oxidation reaction which proceeds at neutral pH. The latter process requires O_2 and Cu^{++}, and is inhibited by EDTA.

In a previous report,[1] we described the activation of fructose 1,6-diphosphatase (FDPase) by a disulfide exchange reaction with cystamine, and suggested that compounds related to cystamine might serve as physiological regulators. We now wish to report the specific activation of FDPase by reduced coenzyme A or acyl carrier protein. This activation occurs either by an oxidation reaction at pH 7.0 or by a disulfide exchange reaction with the oxidized form of these substances at more alkaline pH.

Materials and Methods.—FDPase was purified from rabbit liver as previously described.[2] Enzyme activity was measured spectrophotometrically at 340 mμ by following the rate of fructose 6-*P* production in the presence of TPN and excess glucose 6-*P* isomerase and glucose 6-*P* dehydrogenase. The assay systems (1 ml) contained 1 mM fructose 1,6-P_2, 2 mM $MnCl_2$, 0.15 mM TPN, 2.0 μg of glucose 6-*P* dehydrogenase, 2.0 μg of glucose 6-*P* isomerase, 0.5 mM EDTA, and either 40 mM glycine buffer, pH 9.1, or 40 mM triethanolamine buffer, pH 7.5. The specific activity of the purified FDPase preparation, measured in the alkaline buffer, was 20 international units per mg of protein.

CoA, glucose 6-*P* isomerase, and glucose 6-*P* dehydrogenase were purchased from the Boehringer Mannheim Corp. D-Fructose 1,6-P_2, Na salt, TPN, oxidized glutathione, D,L-penicillamine, and bovine insulin were purchased from Sigma Chemical Corp. Acetyl CoA, dithiothreitol, and D,L-carnitine were obtained from Calbiochem, and glutathione from the Mann Research Laboratories. 5,5′-Dithio-*bis*(2 nitrobenzoic acid) was obtained from K and K Laboratories. Pantethine was purchased from Mann Research Laboratories. Pantetheine was prepared by reduction at pH 8.5 with an excess of mercaptoethanol, which was then removed by lyophilization after acidification with HCl.

Acyl carrier protein, isolated from *E. coli*, was generously provided by Dr. P. R. Vagelos of the Washington University School of Medicine. The mixed disulfide of CoA and glutathione was a gift of Dr. R. N. Ondarza of the University of Mexico, Mexico City. Oxidized CoA was prepared by oxidation of the reduced compound in air at pH 8.5 and 23°. Sulfhydryl groups were measured spectrophotometrically at 412 mμ by titration with 5,5′-dithio-*bis*(2 nitrobenzoic acid)[3] in 0.1 *M* phosphate buffer, pH 8.0.

For the activation experiments, CoA and other compounds tested were dissolved in the same buffer used to obtain the specific reaction conditions. Unless otherwise indicated, the reaction was carried out at 23° in air in 0.1 *M* Tris-Cl buffer, pH 8.5.

Results.—Activation of FDPase by CoA and acyl carrier protein: When FDPase was incubated at pH 8.5 with CoA or acyl carrier protein, the catalytic activity was increased by approximately fivefold (Fig. 1). The reaction was somewhat more rapid with CoA than with comparable concentrations of acyl carrier protein. The initial rate of activation was dependent on the concentration of the reagent but after six to seven hours the extent of activation was similar for all concentrations of CoA tested. Four equivalents of CoA produced nearly maximal activation, and it is known that the enzyme contains four binding sites for the substrate,[4] as well as the allosteric effector AMP[5] and probably has a tetrameric structure.[6]

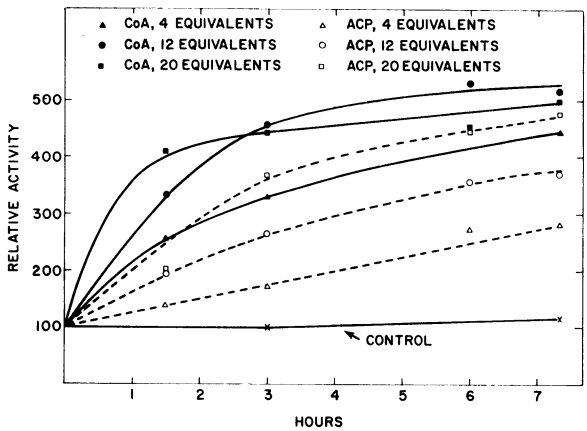

FIG. 1.—Activation of FDPase by CoA and acyl carrier protein. The reaction mixtures, containing 1 mg/ml of FDPase (7.7 μM) in 0.1 *M* Tris-Cl buffer, pH 8.5, were incubated in air at 23° with reduced CoA or acyl carrier protein (ACP), at concentrations of 31 μM (4 equivalents), 93 μM (12 equivalents), or 154 μM (20 equivalents). Aliquots were removed at the times indicated and analyzed for FDPase activity at pH 7.5 as described in the text.

Specificity of the activation reaction: At pH 8.5, oxidized CoA and the mixed disulfide of CoA and glutathione were nearly equally effective. Pantetheine and pantethine showed some activity, and when these were added at higher concentrations (180 equivalents), activation approached that obtained with CoA. Little or no activation was observed with reduced or oxidized glutathione, or other sulfhydryl compounds (Table 1). Insulin, acetyl CoA, and carnitine were also inactive.

TABLE 1. *Specificity of the activation reaction.*

Addition*	Relative activity†	Addition*	Relative activity†
None	100	Acetyl CoA	126
Reduced CoA	516	Reduced glutathione	132
Oxidized CoA	334	Oxidized glutathione	127
Acyl carrier protein	440	Cysteine	132
CoA-glutathione disulfide	463	Dithiothreitol	147
Pantetheine	360	Penicillamine	118
Pantethine	224	Insulin	110
		Carnitine	106

* The conditions were as described in the legend to Fig. 1. Each of the compounds indicated was added in twelvefold excess (93 μM) over FDPase (7.7 μM). Samples were removed for assay after 7 hr at 23°.

† FDPase was assayed at pH 7.5 as described in the text. Specific activity at this pH at the beginning of the experiment was 5.0 units/mg and in the control after seven hours at 23° was 5.9 units/mg.

Effect of pH on the activation reaction: The disulfide exchange reaction with oxidized CoA was observed only under alkaline conditions, above pH 8 (Table 2). No activation was observed with oxidized CoA at neutral pH, although the enzyme can be activated by cystamine at pH 7.5, as previously shown. However, the enzyme can also be activated at neutral pH by reduced CoA in what appears to be an oxidation reaction. This activation by reduced CoA at neutral pH requires atmospheric oxygen, and is completely abolished when the components are incubated under an atmosphere of nitrogen (Fig. 2). The exchange reaction with oxidized CoA is not affected by substituting nitrogen for air in the reaction vessel.

TABLE 2. *Effect of pH on activation of FDPase by reduced or oxidized CoA.*

Addition*	Relative Activity after Incubation at pH 7.0	pH 8.5
None	100†	100†
Oxidized CoA	136	320
CoA-Glutathione disulfide	154	466
Reduced CoA	388	444

* The conditions were as described in the legend to Fig. 1, except that at pH 7.0 the buffer was 0.1 *M* Tris-Cl. The additions, as indicated, were 93 μM reduced CoA, 47 μM oxidized CoA, or 93 μM CoA-glutathione disulfide.

† FDPase activities were measured after 7 hr at pH 7.5, as described in the text.

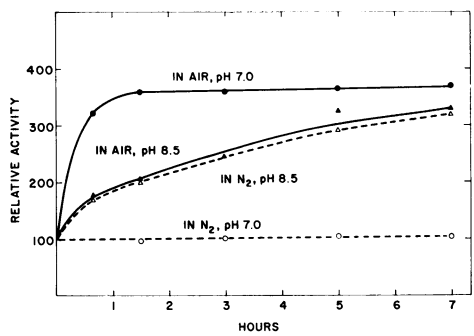

FIG. 2.—Effect of anaerobic conditions on activation by reduced and oxidized CoA. FDPase was incubated in air or in nitrogen with 93 μM reduced CoA (*open* and *closed circles*) or 47 μM oxidized CoA (*open* and *closed triangles*) as described in Table 2.

Activation with reduced CoA at pH 8.5 is probably the result of both oxidation and disulfide exchange reactions since within one hour at this pH CoA is completely oxidized to the disulfide form.

Effect of EDTA and metals: The reaction with reduced CoA at pH 7.0 was found to be inhibited by EDTA, in contrast to the exchange reaction at alkaline pH which was not affected by the addition of chelating agent (Table 3). Inhibition by EDTA was reversed by the addition of a slight excess of Cu^{++}, and to a lesser extent by Fe^{++} ions, which may displace Cu^{++} from the EDTA complex. Other metal ions were without effect.

Since CoA preparations are often contaminated with Cu^{++}, it was necessary to demonstrate that the observed specificity for CoA was not due to the addition of metal ions with this reagent. We found that under similar conditions the addition of Cu^{++} did not result in activation by glutathione or cysteine (Table 3).

TABLE 3. *Effect of EDTA and metals on activation of FDPase.*

Addition*	Incubated† at pH	Relative activity‡
None	7.0	100
Reduced CoA	7.0	433
Reduced CoA + EDTA	7.0	189
Reduced CoA + EDTA + Cu^{++}	7.0	413
Reduced CoA + EDTA + Co^{++}	7.0	161
Reduced CoA + EDTA + Fe^{++}	7.0	207
Reduced CoA + EDTA + Mg^{++}	7.0	157
Reduced CoA + EDTA + Mn^{++}	7.0	189
Oxidized CoA	8.5	306
Oxidized CoA + EDTA	8.5	296
Reduced glutathione + Cu^{++}	7.0	135
Cysteine + Cu^{++}	7.0	135

* The conditions were as described in the legend to Fig. 1. The concentration of EDTA, when added, was 0.1 mM and metals were added at a final concentration of 0.12 mM, except for the last two experiments where the concentration of Cu^{++} was 5 μM. Higher concentrations of Cu^{++} were also ineffective in these experiments.

† The buffer was 0.1 *M* Tris-Cl at either pH.

‡ Assayed after 5 hr at 23°.

Reversal of the activation of FDPase: The results described above suggested that activation of FDPase was associated with the formation of mixed disulfides between enzyme sulfhydryl groups and CoA or acyl carrier protein. Evidence in support of this hypothesis was obtained by the demonstration that the activated enzyme could be restored to its original condition by treatment with glutathione or cysteine (Fig. 3).

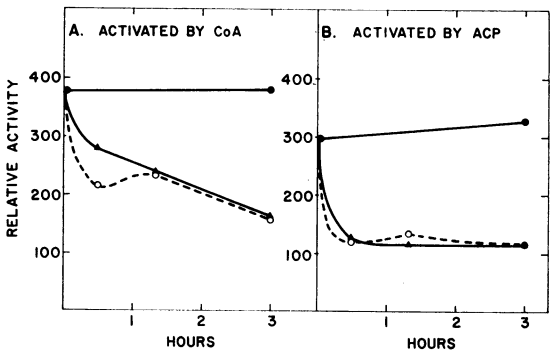

FIG. 3.—Reversibility of the activation of FDPase. FDPase was activated by incubation with 93 μM CoA (*A*) or 93 μM acyl carrier protein (*B*) as described in the legend to Fig. 1. After 6 hr at 23° (zero time in the figure) each reaction mixture was divided into three parts, one of which was incubated without further addition (*closed circles*) while the other two were treated with 5 mM reduced glutathione (*triangles*) or 5 mM cysteine (*open circles*), respectively.

Discussion.—Two mechanisms have been proposed for the regulation of fructose 1,6-diphosphatase.[7] One is the induction of activity at neutral pH when the enzyme normally shows little activity,[8, 9] and a second is the inhibition by the allosteric inhibitor, AMP.[10] We previously have reported that modification of specific cysteine residues in the protein, either by reaction with sulfhydryl reagents,[9, 11, 12] or by a disulfide exchange,[7] increase the activity of the enzyme in the neutral pH range. This activated enzyme is more sensitive to the allosteric inhibitor than is the native enzyme.[1, 7] The observations suggest that modification of sulfhydryl groups may indeed provide a physiological mechanism for control of FDPase activity in glycolysis and gluconeogenesis. The results reported

here implicate two natural compounds, CoA and acyl carrier protein, as possible modulators of FDPase activity.

The fact that the oxidation reaction proceeds readily at neutral pH indicates that the enzyme may contain specific binding sites for the pantetheine derivatives. It is noteworthy that all of the compounds which have been observed to activate FDPase in this manner possess the terminal cysteamine moiety.

The activated form of the enzyme appears to be a derivative in which the activator is linked to the protein by a disulfide bridge. Although the number of groups incorporated has not yet been established, the fact that nearly maximal activation is obtained when the protein is treated with four equivalents of CoA indicates that no more than one mole per active (or allosteric) site is required. Studies of the number of groups bound and the nature of the linkage are in progress.

No information is yet available as to whether these compounds function in the activation of FDPase *in vivo*. Under conditions of gluconeogenesis, acyl carrier protein is more likely to be present in the free form than is CoA, which would be present largely as acyl CoA derivatives. This, together with the fact that the reaction with acyl carrier protein is more rapidly reversed by physiological reducing agents, would support such a role for this compound. However, acyl carrier protein has not yet been detected as such in animal tissues.[13, 14] Final conclusions as to the physiological significance of this reaction await the results of further studies.

Abbreviations: FDPase, fructose 1,6-diphosphatase; CoA, Coenzyme A.

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