SEARCH FOR PHOSPHORYLATED FORMS OF FATTY ACID SYNTHESIS ENZYMES IN THE LIVING ANIMAL

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1. Introduction

The number of enzymes for which a phosphorylated or a nonphosphorylated form has been shown to exist, grows each year. Already including phosphorylase and glycogen synthetase [1,2], pyruvate dehydrogenase [3–7], it has been added to by acetyl CoA carboxylase which has been found to exist in both a phosphorylated inactive form and a non-phosphorylated active one [8,9]. All of these findings have been made in the same general way, i.e. by having ATP act on a cellular fraction with a kinase protein present. It seemed to us of interest, before attempting more specific experiments on tissue preparations, to evaluate the comparative importance of the phosphorylation of synthetase and acetyl CoA carboxylase in the living animal. We therefore administered $^{32}PO_4HNa_2$ to living rats; then isolated, purified and measured the radioactivity of the synthetase and carboxylase of liver. Thus we are able to confirm that carboxylase can be phosphorylated under physiological conditions since, as has also been seen in in vitro experiments, the ^{32}P content increases in the course of purification. On the other hand, in the case of fatty acid synthetase, maximum enzymatic activity coincided with an almost total disappearance of the ^{32}P.

2. Methods

Rats which had been fed ad libitum were injected intraperitoneally with $^{32}PO_4Na_2H$ (1 μCi per 10 rats [200 mCi/mM]), the evening before the experiment. They were killed the following day at 8 a.m., i.e., 14 hr after the radioactive injection. The livers were homogenized in 1.5 vol of phosphate–bicarbonate buffer [10]. The 105 000 *g* supernatant obtained after 1 hr of centrifugation of this homogenate was used to purify the carboxylase and the fatty acid synthetase. The acetyl CoA carboxylase was isolated according to the method of Nakanishi and Numa [11], but with the following two modifications: the proteins used for the various purification procedures were those which precipitated between 0 to 25% ammonium sulfate saturation instead of between 0 to 30%; the purification process was stopped after dialysis. The fatty acid synthetase was purified according to the method described by Burton et al. [12]. A part of the proteins of each fraction was precipitated with CCl_3COOH (final concentration 5%). This precipitate was first washed two times with 5% CCl_3COOH, then washed once with chloroform–methanol 2:1, once with alcohol–ether 1:1, and finally, with pure ether. After evaporation of the ether, the various precipitates were weighed, humidified with 0.1 ml of H_2O, and then dissolved in 1 ml of soluene. Their radioactivity was then measured in a liquid scintillation spectrometer. The different polar solvents for each precipitate were then combined and evaporated, and their radioactivity determined. The activity of acetyl CoA carboxylase was measured by the incorporation of $^{14}CO_3H^-$ in acetyl CoA [13]; that of fatty acid synthetase was measured spectrophotometrically by studying the disappearance of NADPH [14].

3. Results

All of the results are shown on the table. It can be seen that the radioactivity of the fraction containing

Table 1
Radioactivity of fatty acid synthetase (FAS) and acetyl CoA carboxylase (ACX) after administration in vivo of $^{32}PO_4HNa_2$

	Total prot. mg	Specific radioact. of prot. ^{32}P cpm/mg		Total radioact. of proteins	Enzym. act. (units) FAS	Enzym. act. (units) ACX	Total radioact. of lipids ^{32}P	
			%					%
105 000 *g* supernatant	3120	140	(100)	463 800	0.063	1.63	914 200	(100)
→ Amm. sulf. 0–25% precipitate	267.75	171	(122)	45 785	–	3.51	288 466	(31.5)
→ $Ca_3(PO_4)_2$ gel supernatant	100.75	47.35	(33.8)	4 770	–	–	12 245	(1.3)
→ $Ca_3(PO_4)$ gel (washing with 33 mM phosphate buffer	118.8	144.2	(103)	17 130	–	–	35 723	(3.9)
↓ ACX before dialysis	30	284	(202)	8 500	–	145.8	–	
↓ ACX after dialysis	28	298	(214)	8 334	–	155.2	–	
→ Amm. sulf. 0–25% suppernatant	2889	144	(102.8)	416 102	0.152	–	649 000	
→ Ammonium sulfate 0–33% supernatant	2200	157.3	(112.3)	346 060	–	–	92 400	(10.1)
→ Ammonium sulfate 0–33% precipitate	478.8	137.2	(98)	6 569	0.450	–	88 570	(9.6)
↓ $Ca_3(PO_4)_2$ gel	294.4	63.7	(45.5)	18 753	0.550	–	74 150	(8.1)
↓ FAS after DEAE-cellulose	35.3	11.9	(8.5)	420	2.40	–	46 2	(0.00005)

Rats were injected intraperitoneally with $^{32}PO_4HNa_2$ (1 mCi for 10 rats) and were killed 14 hr later. The acetyl CoA carboxylase and fatty acid synthetase of liver were purified and the radioactivity of the proteins obtained during the various purification procedures was measured.
Definition of enzyme units: – synthetase: number of μmoles of NADPH oxidized/min/mg of proteins; – acetyl CoA carboxylase: number of μmoles of $NaH^{14}CO_3$ fixed on acetyl CoA/min/mg of proteins.

acetyl CoA carboxylase increases continuously in the course of the purification process. The specific radioactivity of the protein in the 105 000 *g* supernatant fraction, is, in fact, 140 cpm/mg. However, in the 0 to 25% ammonium sulfate precipitate, it rises to 171 cpm/mg, and continues to rise after adsorption of the acetyl CoA carboxylase by the calcium phosphate gel and elution by the phosphate buffer, at which time it reaches 284 cpm/mg. Finally, after dialysis, the radioactivity is 298 cpm/mg.

In the course of the purification process, the enzymatic activity of acetyl CoA carboxylase increases steadily from 1.63 U for the 105 000 *g* supernatant, to 3.51 for the 0 to 25% ammonium sulfate precipitate and finally to 145.8 and 155.2 for the last two steps. The order of magnitude of the purification is therefore about 95.

In the case of fatty synthetase, on the other hand, a diametrically opposite phenomenon can be observed. The specific radioactivity of the supernatant of the 0 to 25% ammonium sulfate precipitate which was used for the purification of the fatty acid synthetase, was 144 cpm/mg, or practically the same as that of the 105 000 *g* supernatant. The radioactivity of the proteins that precipitate at 25 to 33% ammonium sulfate saturation is 137.3 cpm/mg. The elimination of some of the contaminating proteins by the calcium phosphate gel caused the radioactivity to drop to 63.7. Finally, after DEAE cellulose purification, the radioactivity of the fatty acid synthetase fractions is only 11.9. By contrast, the enzymatic activity of synthetase increases in the course of purification from 0.063 U/mg in the supernatant to 2.4 U for entirely purified enzyme.

Measurements were also made of the radioactivity of the lipids accompanying the proteins of each fraction. The greatest radioactivity was found in the lipids accompanying the proteins of the ammonium sulfate precipitate (0–25%) which are richer in phospholipids than those of other fractions. The lipids accompany the proteins throughout the purification process except for the final DEAE cellulose stage during which they practically disappear.

4. Discussion

Fatty acid synthesis is regulated not only by the activity of the enzymes [11,15–19], but also by the concentration of substrates and cofactors, CO_3H^- [20] and ATP in particular [21,22]. The reaction catalyzed by acetyl CoA carboxylase is considered the limiting step of this synthesis in the liver of rats [23–25]. It has been demonstrated in vitro that this enzyme can exist in both an inactive phosphorylated form and an active non-phosphorylated one [8,9]. ATP could, therefore, have two opposite effects on fatty acid synthesis: stimulatory when it is acting as cofactor, inhibitory when it phosphorylates the acetyl CoA carboxylase. The fact that ATP is capable of these opposing actions could explain why, in vitro, it can stimulate or inhibit fatty acid synthesis depending on whether the liver preparations studied come from fasting or from fed animals [22]. It therefore seemed of interest to determine whether acetyl CoA carboxylase could be phosphorylated under physiological conditions. Since the non-phosphorylated form of this enzyme is the active one, optimal conditions for fatty acid synthesis were not required. Accordingly, the animals were allowed free access to food so that the enzymes whose activity is regulated by the phosphorylation–dephosphorylation process, would be present in measurable quantity in both forms. These same experiments were performed with fatty acid synthetase, which had not yet, to our knowledge, been studied to determine whether it existed in aphosphorylated form.

The intention of the present study was not, it should be noted, to find a correlation between phosphorylation and enzyme activity in vivo, which would have been much too complicated a problem. To do it would have required the use of animals in different physiological conditions, which would then have made the changes observed extremely difficult to interpret. The fact is, there might be a relationship, for example, between the amounts of $^{32}PO_4^-$ bound by the enzyme and a change in the concentration of the enzyme, in the capacity of $^{32}PO_4HNa_2$ to convert to ATP, or in the size of the ATP pool. The object of this present study, however, was only to determine whether, under the same physiological conditions, acetyl CoA carboxylase and fatty acid synthetase are both regulated by the phosphorylation–dephosphorylation process. The results show that this is the case only for acetyl CoA carboxylase, whose radioactivity was found to increase in the course of purification,

and not for synthetase, for which a reverse phenomenon was observed.

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