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CHARACTERIZATION OF THE GLYCOGEN SYNTHASE D FOUND IN LIVER OF THE ADRENALECTOMIZED FASTED RATS

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Summary

We have previously shown that the synthase D (UDPglucose:glycogen 4-α-D-glucosyltransferase, EC 2.4.1.11) present in the liver of the adrenalectomized fasted rat was not converted to synthase I by synthase phosphatase from normal animals, suggesting the presence of a non-substrate form of synthase D (Tan, A.W.H. and Nuttall, F.Q. (1976) Biochim. Biophys. Acta 445, 118—130). The enzymatic properties of this synthase D have now been examined. Using optimal assay conditions, the total amount of synthase D activity in the adrenalectomized fasted rats was similar to that of normal fed rats when 1% glycogen was included in the homogenizing buffer. However, the two enzymes appeared to have different affinities for the substrate, UDPglucose and the modifier, glucose-6-*P*. The changes in kinetic properties were not due to differences in glycogen or to a dialyzable modifier in the extracts. Synthase D from adrenalectomized fasted and from normal fed rats was partially purified. After DEAE-cellulose chromatography, modification appeared to have occurred such that the enzyme from the adrenalectomized fasted rat had properties similar to that of the normal fed rat. The enzymes were cold-labile, had different properties from enzymes in the crude extract and they were both converted to synthase I by synthase phosphatase. We conclude from these studies that the phosphorylation site in the synthase is in a flexible region of the protein. Changes in the ability of the synthase D to interact and be dephosphorylated by synthase phosphatase can occur readily in vivo and in vitro. The molecular basis for the modification remains unknown.

The D and I forms of glycogen synthase are referred to in IUB nomenclature as *a* and *b*, respectively.

Introduction

The enzyme, glycogen synthase (UDPglucose:glycogen 4-α-D-glucosyltransferase, EC. 2.4.1.11) has been shown to exist in a phosphorylated form (synthase D) and a dephosphorylated form (synthase I). Synthase I is active in the ionic conditions prevailing in liver, whereas synthase D is active only in the presence of relatively high concentrations of glucose-6-*P* and UDPglucose. This in vitro activity is completely inhibited by physiological concentrations of adenine nucleotides and inorganic phosphate [1]. The inactive synthase D can be converted to the active synthase I by the enzyme, synthase phosphatase. The latter enzyme has been implicated as an important control site in the glycogen pathway of normal animals.

In adrenalectomized and diabetic rats, abnormalities in glycogen metabolism have been found. Mersmann and Segal [2] observed that the enzyme system which converts glycogen synthase to the active form disappeared from livers of adrenalectomized fasted rat. In alloxan-diabetic, fed and fasted animals, Gold [3] also reported a lack of synthase D to I conversion. Using purified synthase D as substrate, we have localized part of the defect in both the adrenalectomized fasted and diabetic animals to a reduced synthase phosphatase activity [4]. In addition we found that the synthase D present in these animals was not converted to synthase I by the synthase phosphatase from normal rats, suggesting the presence of a non-substrate form of synthase D. Regulation of glycogen metabolism by glucocorticoid and insulin appears, therefore, to be both on synthase phosphatase and on synthase D substrate.

In this study, the enzymatic properties of synthase D in the adrenalectomized fasted animals were examined in the crude extract and compared to those of the normal fed animal. The two enzymes were partially purified and their activity characteristics as well as their suitability as substrate for synthase phosphatase were measured.

Materials and Methods

UDPglucose, glucose-6-*P*, shellfish glycogen and rabbit liver glycogen (Type III), were from the Sigma Company. Rabbit liver glycogen used in the enzyme assay was further purified by passage through a mixed bed ion-exchange resin (Amberlite MB-3). Shellfish glycogen was precipitated two times from 66% ethanol before use. DE-52, in preswollen form was from Whatmann Company. UDP[^{14}C]glucose was prepared by a new method developed in this laboratory [5]. All other chemicals and biochemicals were of the highest grade available from commercial sources.

Male Sprague-Dawley rats (180—250 g) were used. Bilateral adrenalectomy was performed 5—8 days before the animals were killed. Adrenalectomized animals were maintained with 0.9% NaCl in the drinking water. When animals were fasted, the length of fasting was 48 h.

Standard assay of glycogen synthase activity. The assay mixture contained: 4.4 mM UDP[^{14}C]glucose, 6.7 mg/ml column-treated rabbit liver glycogen, 16.7 mM KF, 13.3 mM EDTA, 33.3 mM Tris-HCl (pH 8.8), with and without 7.2 mM glucose-6-*P*. Radioactive glycogen formed was measured using the

method of Thomas et al. [6]. 1 unit of activity is defined as 1 μmol UDP-glucose incorporated into glycogen/min at 30°C.

Partial purification of synthase D from livers of normal fed and adrenalectomized fasted rats. The procedure used was modifed from that of Sanada and Segal [7]. Liver extracts were prepared in 0.25 M sucrose, 0.1 M NaF, 5 mM EDTA, 10 mM mercaptoethanol (pH 7.2). After centrifuging at 78 000 × *g* for 60 min, solid $(NH_4)_2SO_4$ was added to the supernatant to a concentration of 20 g/100 ml. The $(NH_4)_2SO_4$ precipitates were dissolved in 50 mM glycylglycine (pH 7.2)/25% glycerol, 1 mM EDTA/20 mM NaF/10 mM mercaptoethanol (column buffer) with 1% shellfish glycogen added and exhaustively dialyzed. They were then added to a DEAE-cellulose column (2.5 × 35 cm) and eluted with column buffer containing different concentrations of NaCl. Fractions with synthase activity were concentrated and stored in 0.25 M sucrose/50 mM glycylglycine/2 mM dithiothreitol (pH 7.2) at −20°C.

Other methods. Synthase D was purified from rabbit liver by the method of Lin and Segal [8]. The specific activity of the purified synthase D was between 4—10 units/mg protein and the enzyme was free of glycogen, phosphorylase *a*, phosphorylase *b*, synthase phosphatase and phosphorylase phosphatase. Human salivary α-amylase was obtained by the method of Bernfeld [9], up to the second $(NH_4)_2SO_4$ precipitation step. Plasma glucose was determined by the method of Nelson [10]. Protein was measured by the method of Zak and Cohen [11] using bovine serum albumin as standard. Statistical analysis of data was done using the Student's *t*-test for unpaired variates.

Results

Synthase phosphatase and the substrate form of synthase D in the liver of the adrenalectomized fasted rat

Adrenalectomized fasted animals characteristically had very low blood glucose compared to normal fed and fasted, and adrenalectomized fed animals (Table I). Their liver weight expressed as percent of body weight was also greatly decreased. Synthase phosphatase activity in the liver extracts of the normal fed and fasted, and adrenalectomized fed animals was high and over 80% of the synthase D present was converted to synthase I after 60 min of incubation. Under the same conditions, less than 6% of synthase D in liver extracts of adrenalectomized fasted animals was converted. This is due partly to the greatly decreased synthase phosphatase observed in these animals [4]. The amount of synthase D in the substrate form in adrenalectomized fasted rats was estimated in liver extract supplemented with synthase phosphatase from normal animals. Over 90% of the synthase D present was found not to be a good substrate for the synthase phosphatase.

Enzymatic characteristics of glycogen synthase D in crude liver extracts

(a) Effect of glycogen in the homogenizing buffer. Using standard conditions for preparing extracts there appeared to be a decrease in the total synthase measured per liver in the adrenalectomized fasted animals (Table I). However, when 1% glycogen was included in the homogenizing buffer, the total synthase calculated per liver was similar to that of the normal fed rat (Table II).

TABLE I

TOTAL SYNTHASE, SYNTHASE PHOSPHATASE AND SUBSTRATE FORM OF SYNTHASE D IN NORMAL AND ADRENALECTOMIZED RATS

A lobe from each liver was homogenized (a) 1:12 in 100 mM KF/10 mM EDTA (pH 7.0) or (b) 1:3 in 50 mM glycylglycine/10 mM Na_2SO_4/1% glycogen (pH 7.4) and centrifuged at 8000 × *g* for 10 min. The 1:12 extracts were assayed directly for synthase activity with and without glucose-6-*P*. The 1:3 extracts were used for the measurement of synthase D conversion to synthase I as described in [4]. Endogenous synthase phosphatase activity represents the percentage of synthase D in extract converted to synthase I after 60 min of incubation with endogenous synthase phosphatase. Synthase D (substrate form) represents the percentage of synthase D converted to synthase I after 60 min of incubation of extracts which had been supplemented with synthase phosphatase from normal fed animals. Number of animals is in the parentheses. Results are mean ± S.E. Adx, adrenalectomized.

	Blood glucose (mg/100 ml)	Liver (% of body weight)	Synthase		Endogenous synthase phosphatase activity	Synthase D (substrate form)
			units/g	%I		
Normal fed (7)	107 ± 6	4.29 ± 0.1	1.72 ± 0.1	5 ± 0.8	94 ± 3	76 ± 4
Normal fasted (7)	97 ± 12	3.22 ± 0.1	1.58 ± 0.2	9 ± 3	94 ± 4	68 ± 5
Adx fed (6)	87 ± 11	3.39 ± 0.2	1.41 ± 0.1	6 ± 2	83 ± 4	67 ± 10
Adx fasted (8)	57 ± 4	2.70 ± 0.04	1.41 ± 0.1	2 ± 0.2	6 ± 1	10 ± 2

Synthase activity was measured in homogenates as well as extracts prepared with and without glycogen. More activity was found associated with the organelles removable by centrifugation at 8000 × *g* for 10 min in the adrenalectomized fasted animal than in normal fed animals. Addition of glycogen solubilized this activity and more enzyme could be detected in the extract. The reason for this observation is not clear. Glycogen did not appear to influence enzyme stability or enzyme activity since the addition of glycogen immediately after the preparation of extract had no effect (data not shown).

The amount of synthase in the I form measured at pH 8.8 was low in all the animals, with moderately higher activity in normal fasted rats and moderately lower activity in the adrenalectomized fasted animals (Table I). No difference in the percent of synthase in the I form was found when glycogen was included in the homogenizing buffer.

(b) Kinetic properties of glycogen synthase D. Synthase D in liver extracts of the adrenalectomized fasted rat exhibited a narrow pH profile with optimal activity occurring at pH 8.4—9.0. The enzyme had an absolute dependency on

TABLE II

EFFECT OF GLYCOGEN IN HOMOGENIZING BUFFER ON TOTAL SYNTHASE ACTIVITY

A lobe of each liver was homogenized 1:12 in buffer A: 100 mM KF/10 mM EDTA (pH 7.0) or buffer B: 100 mM KF/10 mM EDTA/1% glycogen (pH 7.0). Part of the homogenate was centrifuged at 8000 × *g* for 10 min to give extracts. Activity is expressed as units/g wet weight. Adx, adrenalectomized.

Treatment	Extract		Homogenate	
	A	B	A	B
Normal fed	1.52	1.97	2.03	2.20
Adx fasted	1.43	2.84	3.00	2.95

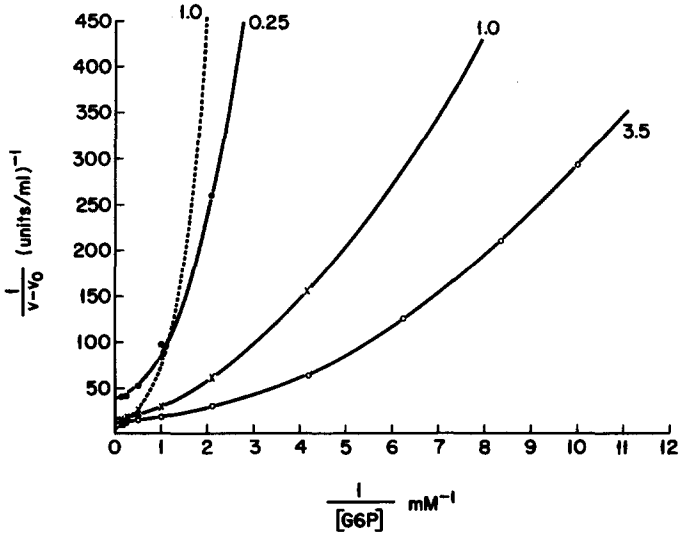

Fig. 1. Dependence of synthase D from liver extract of adrenalectomized fasted rats on glucose-6-*P* for activity. Extract was prepared in 100 mM KF/10 mM EDTA (pH 7.0). The concentration of glucose-6-*P* was varied at fixed concentrations of UDPglucose as indicated: ●———●, 0.25 mM; X———X, △———△, 1.0 mM; and ○———○, 3.5 mM. Assay was done at pH 7.8 (solid lines); or at pH 8.8 (dotted line).

the modifier, glucose-6-*P*, for activity. The affinity of the enzyme for glucose-6-*P* was measured at different fixed concentrations of UDPglucose (Fig. 1). Non-linear Lineweaver-Burk kinetics were obtained. Hill plots of the data yielded $n > 1$, suggesting positive cooperativity between the binding sites for glucose-6-*P*. The concentration of glucose-6-*P* required for half-saturation of the enzyme decreased as UDPglucose concentration was increased suggesting interaction between the two sites. At pH 8.8, the affinity for glucose-6-*P* was lower than at pH 7.8 but the *V* was higher. When the concentration of UDP-glucose was varied at fixed concentrations of glucose-6-*P*, linear relationships in the inverse plot were obtained. The effect of glucose-6-*P* was found to be on the binding of UDPglucose with little or no change in the *V*.

(c) Comparison of the kinetic parameters of synthase D from the liver extracts of normal fed and adrenalectomized fed and fasted animals. Synthase D from the normal fed animals had a similar pH profile as that of the adrenalectomized fasted animals. Subsequent determinations were done at pH 8.5. The affinity of synthase D for UDPglucose and glucose-6-*P* in extracts from the various animals is shown in Table III. The apparent K_m of synthase D for UDP-glucose was determined at low and at saturating concentrations of glucose-6-*P*. They were similar in extracts from normal and adrenalectomized fed rats but significantly increased in the adrenalectomized fasted rats. The $S_{0.5}$ and n of synthase D for glucose-6-*P* at a saturating concentration of UDPglucose in extracts from adrenalectomized fasted rat were also found to be significantly increased compared to those in normal and adrenalectomized fed controls.

(d) Small molecular modifiers of synthase D in liver extracts. Extracts used in studying enzyme kinetics were liver diluted 1 : 50 to 1 200-fold. Thus, the

TABLE III

KINETIC PARAMETERS OF SYNTHASE D FROM LIVER EXTRACTS OF NORMAL FED AND ADRENALECTOMIZED FED OR FASTED ANIMALS

Liver extracts were prepared in 100 mM KF/10 mM EDTA/1% glycogen (pH 7.0). The range of UDP glucose (UDPG) used was 0.24—11.4 mM and 0.12—5.7 mM for the measurement of K_m at low and high concentrations of glucose-6-*P* (G6*P*) respectively. The K_a for glucose-6-*P* was determined at 6.7 mM UDP glucose. Assay was done at pH 8.5. Number of animals used is in the parentheses. Results are mean ± S.E.

Treatment	$K_{m,app.}$ UDPG (mM)		$K_{a,app.}$ G6*P*	
	0.4 mM G6*P*	7.2 mM G6*P*	$S_{0.5}$ (mM)	*n*
Normal fed	1.5 ± 0.06 (3)	0.28 ± 0.006 (4)	0.42 ± 0.055	1.3 ± 0.04 (8)
Adx fed	1.8 ± 0.15 (3)	0.29 ± 0.016 (4)	0.47 ± 0.072	1.4 ± 0.05 (5)
Adx fasted	3.6 ± 0.42 (4) *	0.40 ± 0.015 (7) *	0.77 ± 0.037 *	1.6 ± 0.05 (8) *

* $P < 0.01$ from normal fed and adrenalectomized (Adx) fed.

differences detected between the synthase D of normal fed and adrenalectomized fasted animals should not have been the result of the differential presence of small molecular modifiers of synthase D. To test this further, extracts were exhaustively dialyzed, treated with anion resin, precipitated with $(NH_4)_2SO_4$ and dialyzed. The synthase D in adrenalectomized fasted rat remained in the non-substrate form and had consistently lower affinity for substrate and modifier compared to that of normal fed rats (results not shown).

Partial purification of synthase D from liver extracts of normal fed and adrenalectomized fasted rats

Normal fed rather than normal fasted rats were chosen since extracts from the fasted animals contained a significant amount of synthase I which could not be separated from the synthase D by the method employed. Since the majority of the synthase in the extract of a fed animal was bound to glycogen, an α-amylase treatment step was added in order that the same purification procedure could be used for the two animal preparations. Table IV summarized the results of a representative synthase D preparation from adrenalectomized fasted animals. An enzyme with a spec. act. of 1 was usually obtained, representing a 40—50-fold purification and a recovery of 30—40%.

TABLE IV

PARTIAL PURIFICATION OF SYNTHASE D FROM LIVERS OF ADRENALECTOMIZED FASTED RATS

Starting material: Livers from eight adrenalectomized fasted rats.

	Volume (ml)	Total activity (units)	Total protein (mg)	Specific activity (units/mg)
Extract	128	80	3920	0.02
78 000 × *g* supernatant	114	53	2820	0.018
$(NH_2SO_4$ precipitate	50	33	475	0.069
DEAE-cellulose	100	25	31	0.81

Substrate suitability and other properties of the partially purified synthase D

It is possible to convert essentially all of the endogenous synthase D to synthase I in the crude extract of normal animals as shown in Table I. Exogenous synthase D purified from normal fed rabbit was a good substrate for synthase phosphatase but the maximal amount which could be converted to synthase I varied from preparation to preparation, ranging from 60 to 80%. Synthase D prepared from normal fed rats by the present procedure was also only partially convertible to synthase I. The average amount converted was about 60% of the total synthase D activity present, as can be seen in Table V. In the crude extract from adrenalectomized fasted rat, only a very small fraction of synthase D was convertible to synthase I. However, after partial purification, 54% of the synthase D obtained could be converted to the I form.

On closer examination, many properties of enzymes were found to be altered during purification. Synthase D, which usually had the highest activity at pH 8.8, was more active at pH 7.8 after the DEAE-cellulose chromatography. The enzyme which was strongly inhibited by 10 mM phosphate was no longer inhibited by this metabolite. As can be seen later, changes in kinetic properties had also occurred.

Effect of preincubation on the enzymatic properties of partially purified synthase D

We found that enzyme stored frozen after the DEAE-cellulose column step was partially cold-inactivated. About a 2-fold increase in activity could be obtained after 1—2 h of preincubation. However, after a prolonged period of storage it was no longer possible to activate the enzyme.

The enzyme was found to have different properties with and without preincubation. Without preincubation, the enzyme had a pH profile with an optimum at pH 7.5 (Fig. 2). After preincubation a form with higher activity at pH 8.5 was observed. Synthase D in crude extracts was strongly inhibited by ATP and inorganic phosphate at pH 7.8. After partial purification, the synthase D was no longer inhibited. Preincubation brought about the return of a form

TABLE V

SUBSTRATE SUITABILITY AND KINETIC PARAMETERS OF PARTIALLY PURIFIED SYNTHASE D FROM NORMAL FED AND ADRENALECTOMIZED FASTED RATS

Partially purified enzymes diluted in 100 mM KF/10 mM EDTA/1 mM dithiothreitol (pH 7.0) were either kept on ice or preincubated at 25°C for 80 min. Assay conditions for kinetic studies were the same as in Table III. Synthase D (substrate form) represents the % of synthase D converted to synthase I after 60 min of incubation with synthase phosphatase from extract of normal fed animals. Data represent means from two experiments. UDPG, UDPglucose; G6*P*, Glucose-6-*P*; Adx, adrenalectomized.

Treatment	Synthase D (substrate form)	Pre-incubation	$K_{m,app.}$ UDPG		$K_{a,app.}$ G6*P*	
			0.4 mM G6*P*	7.2 mM G6*P*	$S_{0.5}$ (mM)	*n*
Normal fed	60	—	2.6	0.62	0.37	0.92
		+	1.7	0.21	0.31	1.3
Adx fasted	54	—	6.1	0.86	0.38	1.2
		+	2.2	0.24	0.48	1.6

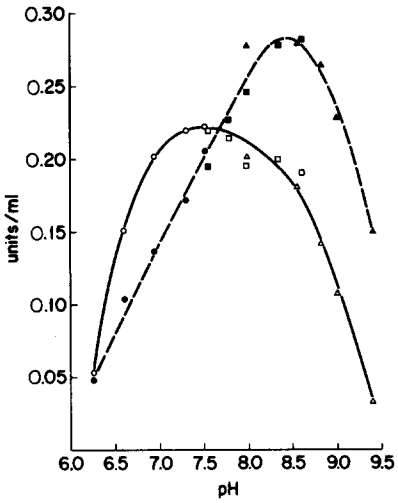

Fig. 2. Effect of preincubation on pH profile of a partially purified preparation of synthase D from adrenalectomized fasted rats. Enzyme which had been stored frozen at a concentration of 4—8 units/ml was diluted to 0.5 units/ml with 100 mM KF/10 mM EDTA/1 mM dithiothreitol (pH 7.0). The diluted enzyme was either kept on ice (open symbols) or preincubated for 80 min at 25°C (solid symbols). Standard assay conditions were used except pH determination was done in imidazole buffer (○———○), glycyclglycine (□———□) or Tris-HCl (△———△). To minimize activation of enzyme during assay, time was reduced to 5 min.

which was sensitive to these metabolites. Synthase D prepared from normal fed rat exhibited similar behavior under these circumstances.

After partial purification the difference in the kinetic properties of the enzymes from normal fed and adrenalectomized fasted animal had largely disappeared (Table V). When compared to the enzymes in the crude extracts, synthase in the non-incubated samples had higher K_m for UDPglucose and had lost some cooperativity for glucose-6-*P* even though the $S_{0.5}$ was not significantly changed. After preincubation, the kinetic parameters were similar to the values found for the enzyme in the crude extract of normal fed rat.

Discussion

Our results indicate that there is a modified form of synthase D in liver of the adrenalectomized fasted rat. The enzyme is altered in such a way that its phosphate moiety is no longer accessible to synthase phosphatase and thus it can not be converted to synthase I. This modification still permitted the enzyme to be fully active at saturating concentrations of substrate and modifier. It still had the same pH profile and was inhibited by ATP and inorganic phosphate. Kinetic measurements, however, indicated that it had a reduced affinity for UDPglucose and glucose-6-*P*.

The presence of different kinetic forms of synthase D in crude extracts had been reported in several tissues associated with different metabolic states. Adipose tissue isolated from fasted rats appeared to contain a kinetic form of synthase D different from that obtained from fed rats [12]. When human

leukocytes were incubated with glucose, a form of synthase, different kinetically from either the D or I was believed to be responsible for the glycogen synthesized [13]. Glycogen deposited in muscle after exercise was also suggested to be due to a different kinetic form of synthase [14]. However, in all these systems where the interconversion enzymes are present, it is extremely difficult to distinguish enzyme forms since mixtures of the classical D and I forms give rise to complex kinetics. The various forms can be defined only after they have been separated and characterized.

Many forms of muscle synthase, other than the D and I forms, have been obtained in vitro. One form was produced by a limited tryptic digestion of the synthase I in which a C-terminal peptide was removed. It retained enzyme activity but only in the presence of glucose-6-*P* [15]. Purified muscle synthase I can be phosphorylated at different positions and in various amounts by cyclic AMP-dependent and AMP-independent kinases. The resultant products are all cyclic active enzymes but have different kinetic properties [16—20]. In addition, synthase D itself has been suggested to undergo further phosphorylation to become an even less active species [21]. So far it has been difficult to attribute physiological significance to these multiple forms of synthase since their presence has only been demonstrated in vitro.

The molecular basis of the modification of liver synthase D in the adrenalectomized fasted rat is not clear. It is possible that the synthase present in the adrenalectomized fasted animal in vivo is a proteolytic product of the normal synthase D or is an under- or over-phosphorylated form of the enzyme. Alternatively, it could be the same as that in the normal animal but because of association with a tightly-bound cofactor or a specific protein factor, its phosphate moiety has become buried and is not accessible to the phosphatase. An attempt was made to isolated the modified form of synthase D from the adrenalectomized fasted rat in order to compare its structure with that of the enzyme from the normal fed animal. We found purification altered the enzyme from the adrenalectomized fasted animal such that it had properties similar to that of the normal fed animal. Both enzymes were found to be cold-inactivated, had different properties from enzymes in the crude extracts and they were both converted, to synthase I by synthase phosphatase. This could be interpreted as, partial purification via DEAE-cellulose chromatography has removed a tightly-bound factor from the synthase D of the adrenalectomized fasted rat, such that it is the same as the synthase D from the normal fed rat.

However, our experience with purified rabbit liver synthase D indicated that the enzyme conformation can be modified easily by in vitro manipulations. Using essentially identical procedures for purification, we have obtained preparations which have high spec. act. but which vary greatly in their suitability as substrate for synthase phosphatase. It is possible that the modified form of synthase D from the adrenalectomized fasted and the form from normal fed animal have both been altered during purification such that synthase phosphatase can no longer discriminate between them. More work has to be done to find factors or conditions which can stabilize the site which is dephosphorylated during purification. Only when synthase is isolated in a completely native state will it be possible to identify the molecular basis of modification of synthase D in the adrenalectomized fasted animal. In this regard, X-ray

crystallographic studies of muscle phosphorylase *a* have indicated that the phosphorylation site is in a separate domain from the catalytic site and it is possible to modify the conformation of one site without producing changes in the other [22].

Using different animal preparations, previous studies from our laboratory indicated that both insulin and glucocorticoid were important in the maintenance of synthase phosphatase activity [4]. The loss of synthase D in the substrate form paralleled the decrease in synthase phosphatase. Preliminary studies indicate that the time course of return of synthase phosphatase after giving animals oral glucose is also accompanied by a recovery of synthase to the substrate form. Whether the change in synthase D is dependent on a change in synthase phosphatase is not clear. It could be postulated that binding or close proximity to synthase phosphatase is essential for the maintenance of synthase in the substrate form. The enzyme may become modified when this association is lost as in the adrenalectomized fasted and diabetic fed rat [4]. On the other hand, in human choriocarcinoma cells a change in synthase D substrate form has been observed without a corresponding change in synthase phosphatase [23].

Our studies indicate that regulation of glycogen synthesis is complex. Modification of the enzyme substrate as well as activation of the inter-converting enzymes can occur. It is tempting to extrapolate the observations from the adrenalectomized fasted animals to explain activation of glycogen synthase in the normal animal. Since synthase phosphatase activity normally is high, activation by glucose could involve the conversion of synthase D from a poor substate form to a good substrate form.

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