An altered $T_2\beta$ translocase of the glucose-6-phosphatase system in the membrane of the endoplasmic reticulum from livers of Ehrlich-ascites-tumour-bearing mice

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The inhibitory interactions of orthophosphate (P_i) with the glucose-6-phosphatase system of intact microsomes derived from the livers of normal and Ehrlich-ascites-tumour-bearing mice reveal the appearance of a novel form of the $T_2\beta$ translocase component of the glucose-6-phosphatase system in tumour-stressed mice. Kinetic studies, with and without 20 mM P_i , show a strictly classical competitive inhibition, with a K_{i,P_i} of 4.2 mM, with disrupted microsomes from both control and tumour-bearing mouse liver. Inhibition was also observed with intact

microsomes from livers of control mice, and contributions by both competitive and non-competitive components of inhibition were quantified by calculation of K_{is,P_i} and K_{ii,P_i} values respectively. However, little inhibition was noted with intact microsomes from the livers of tumour-bearing mice. It is concluded that this novel form of $T_2\beta$ is less able to transport P_i from the cytosol to the endoplasmic reticulum lumen, perhaps because of the tumour-related increased K_m for P_i transport in this direction.

INTRODUCTION

The hepatic enzyme glucose-6-phosphatase catalyses the reaction terminal to both glycogenolytic and gluconeogenic processes [1,2]. It is a complex system, consisting of at least six components [3–6]: the enzyme catalytic unit itself, a stabilizing protein (conveying calcium ion sensitivity), and four transport components (each with its own specificity for substrates and/or products). Figure 1 shows a model of glucose-6-phosphatase

structure/function according to this substrate translocase–catalytic unit concept [6].

This multiplicity of glucose-6-phosphatase components provides several opportunities for regulation through metabolic modifiers. Responses of the system to a number of different stresses that enhance gluconeogenesis might arise from any mechanism whereby the enzyme system has changed to accommodate enhanced Glc production. Established responses include: (1) an increase in the catalytic unit, both in terms of protein levels [7] and catalytic activity [8] in untreated diabetes; (2) an increase in transporter (T_1) for glucose 6-phosphate (Glc-6-P) [9,10] and formation of catalytic unit protein with an altered kinetic behaviour [11] in response to pharmacological doses of glucocorticoids; and (3) an increase in the $T_2\beta$ translocase in liver microsomes derived from Ehrlich-ascites-tumour-bearing mice [12]. The last response is expressed in terms of increased immunochemically detectable $T_2\beta$ protein and in its ability (as deduced from kinetic considerations) to transport P_i from the lumen to the cytosol, and to transport the substrates carbamyl phosphate and PP_i into the lumen of the microsome.

The third response is the subject of further studies described here. Two features of glucose-6-phosphatase structure/function are of special note when considering P_i transport. First, P_i , a product of Glc-6-P hydrolysis, is an effective competitive inhibitor of Glc-6-P hydrolysis by the catalytic unit [13]. Secondly, translocase T_2 [10], now believed to be a specific form of T_2 termed $T_2\beta$ [14], is assumed to be a bi-directional translocase of carbamyl phosphate, PP_i and P_i between cellular cytosol and the lumen of the endoplasmic reticulum (see Figure 1). We designed the study described here on the basis of these facts and on the observed increase in $T_2\beta$ protein level and activity in microsomes derived from Ehrlich-ascites-tumour-bearing mice [12]. We expected that intact microsomes derived from the livers of tumour-bearing mice would have an enhanced sensitivity to inhibition of Glc-6-P hydrolysis by exogenously added P_i . The

Figure 1 Structure/function relationships of the glucose-6-phosphatase system according to the current substrate translocase–catalytic unit concept

A cross-section of the endoplasmic reticulum (E.R. MEMBRANE) is depicted. SP indicates the stabilizing protein; T_1 , $T_2\alpha$, $T_2\beta$ and T_3 are substrate/product translocases with the indicated specificity; and the catalytic unit is glucose-6-phosphatase (EC 3.1.3.9) enzyme. Two forms of T_2 with differing specificity, termed $T_2\alpha$ and $T_2\beta$, have been proposed [14,15]. Modified from Foster et al. [6].

results showed that the opposite situation prevails. This revised our thinking and clearly supported a model of glucose-6-phosphatase (Figure 1) that can respond to stress by reconfiguring or altering the T_{β} translocase component.

MATERIALS AND METHODS

Mice, microsomal preparations, sources of chemicals, and methods were as described in ref. [12]. Incubation mixtures, pH 7.0, contained, in 0.3 ml of 40 mM Hepes buffer, 10–25 μ g of microsomal protein and 1.0, 1.2, 1.4, 1.8, 2.5, and 10 mM Glc-6-P. P_i , when present, was at a concentration of 20 mM. Incubations were for 6 min at 30 ± 0.1 °C. The production of Glc from Glc-6-P was measured [16]. Standard curves of 0–100 nmol of Glc were prepared concurrently with each experiment.

Classic reversible inhibition studies were performed with microsomes obtained from the livers of control and Ehrlich-ascites-tumour-bearing mice. Microsomes were either supplemented with Triton X-100, to 0.06% (w/v) ('disrupted microsomes'), or were supplemented with an equal volume of water [12]. In the latter case, all activities were adjusted [17] for the disrupted component of untreated microsomal preparations ('intact microsomes'). Microsomes from two control or two tumour-bearing mice were combined. Assays were carried out with six pooled control preparations and five pooled preparations from tumour-bearing mice.

Inhibitor constants (K_{ii} and K_{is}) were calculated [18,19] from kinetic data. K_{ii} values were calculated on the basis of y -axis intercepts. They are presumed to quantify the non-competitive component. K_{is} values were calculated on the basis of the slopes

of double-reciprocal plots generated in the absence and presence of P_i . They are presumed to quantify the competitive component of inhibition in a complex system such as that with intact microsomes, where elements of both may be manifest [18,19]. Protein determinations were by the Pierce bicinchoninic acid method [20]. Data are presented as mean $\pm$ S.E.M. values determined by the analysis of variance [21].

A significant difference is assumed to occur when $P < 0.05$.

RESULTS

Typical results obtained with individual pooled preparations are presented as Lineweaver–Burk double-reciprocal plots [22] in Figure 2. Inhibitions by P_i were of the classical, reversible competitive type [23] in disrupted microsomal preparations from both control (Figure 2c) and tumour-bearing mice (Figure 2d). Inhibition observed with P_i was of the 'mixed' type [24] in intact microsomal preparations derived from control mice (Figure 2a). Quite closely spaced, parallel lines were obtained with and without 20 mM P_i using intact microsomes from tumour-bearing mice; this is indicative of no competitive inhibition and little overall inhibition (Figure 2b).

Results obtained from all pooled microsomal preparations were analysed kinetically to distinguish and quantify the relative contributions made by competitive and non-competitive inhibition to each preparation [18,19]. In intact microsomes, both the substrate, Glc-6-P, and the inhibitor, P_i , must be transported by specific translocase actions to the lumen of the microsome where the catalytic unit is sequestered. Purely competitive inhibition by P_i versus Glc-6-P is to be anticipated with disrupted

Figure 2 Glc-6-P hydrolysis in the presence (○) and absence (●) of exogenously added 20 mM P_i .

Data with intact microsomes from control and tumour-bearing mice are indicated in (a) and (b) respectively. Data with disrupted microsomes from control and tumour-bearing mice are given in (c) and (d) respectively. Data are plotted in double-reciprocal fashion [22–24]. Units for $1/v$ and for $1/[Glucose-6-P]$ are $(\mu\text{mol/min per mg of protein})^{-1}$ and $(\text{mM})^{-1}$ respectively.

Table 1 P_i inhibition of glucose-6-phosphatase hydrolysis by intact and disrupted hepatic microsomes derived from control and tumour-bearing mice

K_{ii,P_i} and K_{is,P_i} values were calculated as described in the text. The $K_{is,P_i}/K_{ii,P_i}$ values are dimensionless.

Experimental group	Intact microsomes			Disrupted microsomes		
	K_{ii,P_i} (mM)	K_{is,P_i} (mM)	$K_{is,P_i}/K_{ii,P_i}$	K_{ii,P_i} (mM)	K_{is,P_i} (mM)	$K_{is,P_i}/K_{ii,P_i}$
Control ($n = 6$)	48.4 ± 7.4	35.3 ± 1.5	0.729 ± 0.116	$150 \pm 14^{**}$	$4.31 \pm 0.10^{**}$	$0.030 \pm 0.003^{**}$
Tumour bearing ($n = 5$)	31.7 ± 3.9	0^*	0^*	$60.6 \pm 7.6^{**}$	$4.04 \pm 0.22^{**}$	$0.071 \pm 0.009^{**}$

* $P < 0.05$ for tumour-bearing versus Control groups; ** $P < 0.05$ for disrupted microsomes versus intact microsome group.

microsomes where transport is not a factor [13], but purely competitive inhibition cannot occur with intact microsomes [19]. Both K_{is,P_i} values (inhibitor constants that quantify the amount of competitive inhibition), and K_{ii,P_i} values (which quantify the non-competitive component of observed inhibition) were calculated. These values are presented in Table 1, along with calculated $K_{is,P_i}/K_{ii,P_i}$ ratios. The latter gives an index of the competitive relative to the non-competitive inhibitory components [18,19]. Note that the smaller the value for the ratio, the greater is the contribution by competitive-type inhibition.

The K_{ii,P_i} (for non-competitive inhibition) was very high, the K_{is,P_i} (for competitive inhibition) was much lower, and the $K_{is,P_i}/K_{ii,P_i}$ ratio was very small (Table 1); all are indicative of competitive inhibition by P_i of Glc-6-P hydrolysis by disrupted microsomes (i.e. by the catalytic unit of the glucose-6-phosphatase system). These observations are consistent with kinetics experiments illustrated in Figures 2(c) and 2(d). The K_{is,P_i} values determined with disrupted microsomes derived from control and tumour-bearing mice were indistinguishable (Table 1).

Quite different results were obtained when intact microsomes from tumour-bearing and control mice were studied for glucose-6-phosphatase activity. K_{is,P_i} was infinite with preparations from tumour-bearing mice, as was the $K_{is,P_i}/K_{ii,P_i}$ ratio. This indicates the absence of competitive inhibition of glucose-6-phosphatase activity by P_i in intact microsomes from tumour-bearing mice. The K_{ii,P_i} value indicates only a small contribution by non-competitive inhibition. Aspects of both competitive and non-competitive inhibition of glucose-6-phosphatase by P_i were seen with intact microsomes from control mice (Table 1). The K_{is,P_i} (indicative of competitive inhibition) is about 73 % of that for the K_{ii,P_i} (non-competitive inhibition).

DISCUSSION

Our results point to an altered or reconfigured $T_2\beta$ translocase in the membrane of the hepatic endoplasmic reticulum of Ehrlich-ascites-tumour-bearing mice. We will call this altered component $T_2\beta^*$. The train of reasoning that leads to this conclusion starts with our earlier observations [12] showing that the response to Ehrlich ascites tumours involves both an increase in $T_2\beta$ protein and modifications in the translocase's behaviour. These changes enhance transport of P_i , a product of Glc-6-P hydrolysis, from the microsomal lumen, lowering its concentration there [12]. This would lead to enhanced hydrolysis of luminal Glc-6-P in liver microsomes derived from tumour-bearing mice because P_i typically is a competitive inhibitor [13] of this process. {A mechanism explaining the tumour-related increase in the apparent K_m for Glc-6-P [12] on the basis of impaired P_i efflux from microsomes, with consequent increase in intraluminal $[P_i]$ leading to an increase in apparent K_m for Glc-6-P due to competitive inhibition, might be thought of as an alternative to an enhanced

efflux of P_i as suggested earlier [12]. However, this is contradicted by several experimental observations with intact microsomes from tumour-bearing compared with control mice: (a) the percentage latency of Glc-6-P phosphohydrolase activity decreases (see Table 3 in [12]); (b) the apparent V_{max} for Glc-6-P hydrolysis increases (see Table 1 in [12]); (c) the percentage latency for PP_i , phosphohydrolase activity decreases extensively (see Table 3 in [12]); and (d) the apparent K_m for carbamyl phosphate decreases nearly threefold (see Table 3 in ref. [12]). All of these tumour-related responses are consistent with an enhanced removal of P_i from the microsomal lumen, and all are inconsistent with a lowered removal of luminal P_i . The desensitization of the glucose-6-phosphatase system to inhibition by exogenous P_i with intact microsomes, as reported here, fits as a complementary part of this regulatory response mechanism. Lessened uptake of P_i through $T_2\beta^*$ existence in the tumour-bearing mouse would augment the reduction in intraluminal P_i concentration brought about by enhanced transport of P_i from the lumen to the cytosol. The response in $T_2\beta^*$ in microsomes from tumour-bearing mice is then doubly effective in enhancing Glc-6-P hydrolysis at the $T_2\beta$ location, even in the absence of change in the catalytic unit or Glc-6-P translocase.

The previously published substrate translocase model of glucose-6-phosphatase ([3–6,10], see Figure 1) led us to expect an enhanced rather than a decreased uptake of P_i in intact microsomes from tumour-bearing mice where $T_2\beta$ levels are elevated [12]. The unexpected results, however, reminded us that the depressed uptake and consequent lessened inhibition of Glc-6-P hydrolysis is just what should prevail if adjustments in regulation for the Ehrlich-ascites-tumour-bearing mouse are to have metabolic credibility. Enhanced transport of P_i from the microsomal lumen to the cytosol should indeed be accompanied by a decreased transport of P_i from the cytosol back into the microsome. Only then would a maximal reduction in luminal P_i concentration be achieved for the stressed biological condition represented by the tumour.

The most reasonable mechanistic explanation for the tumour-related changes in the glucose-6-phosphatase system described here and earlier [12] is that multiple forms of translocase $T_2\beta$ exist. One form, which we will call $T_2\beta$ (see Figure 1), is capable of bi-directional transport of P_i and presumably carbamyl phosphate and PP_i . The other, $T_2\beta^*$ is present in tumour-bearing mice and transports carbamyl phosphate and PP_i effectively into microsomes, transports P_i effectively out of microsomes [12] but not into microsomes (this study), and increases in amount in response to the presence of Ehrlich ascites-tumour cells. The $T_2\beta^*$ may replace the $T_2\beta$ form in tumour-stressed mice because normal competitive inhibition of Glc-6-P hydrolysis by exogenous P_i is absent.

A modification resulting in a large increase in the K_{m,P_i} for transport into microsomes by the $T_2\beta^*$ form, with either no

change or possibly a decrease in K_{m,P_i} for transport out of microsomes, seems the most likely explanation for the differences in P_i transport specificity in the two $T_2\beta$ forms. The possibility that T_2 form $T_2\alpha$ (see Figure 1) is involved in the tumour-related response described here is contraindicated by the facts that (a) $T_2\alpha$ is not interactive with the antibody employed [12,14], and (b) it is believed that the K_{m,P_i} for transport of P_i from the microsomal lumen to the cytosol is probably too big to support significant interactions with the near-physiological P_i concentration that we used [12,14,15].

This concept of multiple forms of a component of the glucose-6-phosphatase system has precedent. Traxinger and Nordlie [11], showed the formation of glucose-6-phosphatase enzyme, but with lower specific activity than normal, in response to glucocorticoid administration. More recently Allan et al. [25] have found different forms of the glucose-6-phosphatase enzyme with different substrate specificities in cell lines. Evidence also exists for two different forms of T_1 (the endoplasmic reticulum Glc-6-P translocase) that respond differently to fatty acyl-CoA esters [26]. Molecular mechanisms whereby multiple forms of T_2 with different transport capacities may arise include (a) changes in the isoform of T_2 that is expressed, (b) variations arising through non-covalent modifications, or (c) covalent modifications. Evidence for all three types of mechanisms has been reported with other components of the glucose-6-phosphatase system [7–11,27,28].

We have suggested [12] that the increase in apparent K_m value for Glc-6-P, a reflection of the increased differential between microsomal and extramicrosomal Glc-6-P concentrations [29], might serve to maintain a viable cytosolic pool of Glc-6-P in the hepatocyte under conditions of enhanced demand for Glc production. The diminished transport of P_i into the microsomal lumen via $T_2\beta^*$ described here, along with enhanced transport of P_i out of the microsome [12], would increase the efficiency of the mechanism for maintenance of an increased apparent K_m for Glc-6-P.

These changes in the amount of translocase $T_2\beta$ protein, as well as in its kinetic characteristics, can work synergistically to provide the best possible adjustment in the glucose-6-phosphatase system for optimal function under tumour-stressed conditions.

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