The hepatic glucose-6-phosphatase system in Ehrlich-ascites-tumour-bearing mice

Richard W. LUCIUS,* Ian D. WADDELL,† Ann BURCHELL‡§ and Robert C. NORDLIE*

*Department of Biochemistry and Molecular Biology, Ireland Research Laboratory, University of North Dakota School of Medicine, Grand Forks, ND 58202, U.S.A., and Departments of †Child Health and ‡Obstetrics and Gynaecology, Centre for Research into Human Development, Ninewells Hospital and Medical School, Dundee DD1 9SY, Scotland, U.K.

To examine the effects of the presence of Ehrlich ascites tumours on both the catalytic unit and the substrate/product translocase components of the glucose-6-phosphatase system *in vivo*, we isolated microsomes from the livers of control and tumour-bearing mice. Samples were analysed immunochemically for the quantity of catalytic unit, stabilizing protein and translocases T₂ and T₃ proteins. In comparison experiments, a variety of kinetic studies were performed. The most striking findings in tumour-bearing mice were: a 2.5-fold increase in the quantity of translocase T₂ protein; increases in the *K_m* and *V_{max}* for glucose

6-phosphate phosphohydrolase; and a decrease in the *K_m* value for carbamoyl phosphate (carbamoyl-*P*) of carbamoyl-*P*:glucose phosphotransferase, all with intact microsomes. The percentage latency at *V_{max}* decreased for PP_i phosphohydrolase and for glucose 6-phosphate phosphohydrolase, but was unaffected for carbamoyl-*P*:glucose phosphotransferase. These observations support a tumour-related increase in translocase T₂ capacity *in vivo*, as it transports P_i from the microsomal lumen to the medium and carbamoyl-*P* or PP_i from the medium to the microsomal lumen.

INTRODUCTION

Glucose-6-phosphatase (D-glucose-6-phosphate phosphohydrolase; EC 3.1.3.9) is involved in gluconeogenic and glycogenolytic metabolism [1,2]. The multifunctional capabilities of hepatic glucose-6-phosphatase [2,3] are demonstrated by the enzyme's ability to catalyse several hydrolytic and biosynthetic reactions:

In the liver, the glucose-6-phosphatase system is composed of at least five discrete protein components: a catalytic unit, stabilizing protein, and three individual substrate-specific translocase proteins [4–7]. Five variants of Type 1 glycogen-storage disease [8–12] have also been demonstrated to be due to defects in each of the components of the glucose-6-phosphatase system.

The structure of the microsomal glucose-6-phosphatase enzyme system has not been completely described, as only limited topological information is available (e.g. [13]). The catalytic unit is embedded in the lipid-rich hydrophobic membrane, with its active site on the luminal side of the endoplasmic reticulum (ER), and it is associated with a regulatory Ca²⁺-binding protein. Substrates outside the ER do not have direct access to the catalytic unit. Three substrate-specific ER membrane transport proteins transport Glc-6-*P* (translocase T₁), carbamoyl-*P*, PP_i and P_i (translocase T₂) and Glc (translocase T₃ or GLUT7) across the ER membrane. To date, only T₂ and T₃ have been purified [14–16], but Glc-6-*P* transport has been directly shown to take place across the ER membrane [17], confirming the existence of T₁. Reactions (1)–(3) have been used to study these individual components of the glucose-6-phosphatase system.

The activities of glucose-6-phosphatase vary in response to

different nutritional or hormonal perturbations. Prolonged fasting or experimentally induced diabetes results in an increase in catalytic-subunit activity, with little or no change in the translocases [18–23]. This results in an increase in the *V_{max}* for glucose-6-phosphatase in intact microsomes. It also results in an increase in the apparent *K_m* for Glc-6-*P* and an increased latency of glucose-6-phosphatase activity in intact microsomes [18–23].

The presence of tumours also places a potent gluconeogenic stress on the host [24,25]. We wished to ascertain whether or not the responses of the glucose-6-phosphatase system in Ehrlich-ascites-tumour-bearing mice were identical with previously elucidated responses to experimental diabetes. These tumour-bearing mice were selected as subjects to study because of their availability and their appropriateness for these studies. Shapot and Blinov [24] studied the course of development of hypoglycaemia with tumour growth, using mice bearing Ehrlich ascites carcinoma cells. They concluded that 'the ability of the Ehrlich carcinoma to reduce responsiveness of the host's tissues, affecting gluconeogenesis, may reflect a systemic action of the neoplasm on the distant organs' [24]. We accordingly have employed this model for our studies of tumour-related adaptations of the hepatic enzyme system catalysing the terminal reaction in glucose production. We employed immunochemical techniques to quantify the glucose-6-phosphatase catalytic unit, stabilizing protein and translocases T₂ and T₃ in tumour-bearing and control mice. Kinetic analyses involving reactions (1)–(3) as probes were employed to examine the impact of any changes in individual components of the glucose-6-phosphatase system on its overall activity and its individual kinetic parameters.

MATERIALS AND METHODS

Fine biochemicals, of the highest grade commercially available, were obtained from sources listed previously [26]. Biotinylated anti-sheep antibody and an ECL (enhanced chemiluminescence)

Abbreviations used: ER, endoplasmic reticulum; Glc-6-*P*, glucose 6-phosphate; Glc, glucose.

§ To whom correspondence should be addressed.

detection system were purchased from Amersham International, Amersham, Bucks., U.K. Nitrocellulose was from Schleicher and Schuell, Dassel, Germany.

Preparation of microsomes

Male mice of the HA/ICR strain were originally from Cumberland View Farms, Clinton, TN. The mice were maintained on Purina Rodent Chow and water *ad libitum* under a 12-h light/12-h dark schedule. Ehrlich ascites-tumour cells were introduced into 7-week-old mice by intraperitoneal injection and allowed to grow for 7 days before killing the animals (56 days old). The mice were decapitated and the livers rapidly removed, blotted and weighed. Livers (1.6 ± 0.15 g and 1.8 ± 0.15 g for tumour-bearing and control mice respectively) from two mice were pooled and homogenized in 10 vol. of ice-cold 0.25 M sucrose/5 mM Hepes, pH 7.0. The homogenate was subjected to differential centrifugation to extract the microsomal fraction [27]. The resulting microsomes were resuspended in the buffered sucrose/Hepes solution (1 g of liver wt./ml of solution). Portions were frozen at -20°C for later use. When needed, samples were thawed at 0°C and diluted with ice-cold 0.25 M sucrose before assay. Previous [28] and supplemental studies (R. W. Lucius and R. C. Nordlie, unpublished work) indicate no change in microsomal specific activity, percentage latency and kinetic parameters of glucose-6-phosphatase due to freezing of microsomes followed by a single careful thaw at 0°C and prompt assay under our experimental conditions. Triton X-100 was added to 0.06% (w/v) to disrupt the microsomal membranes. An equivalent amount of ice-cold glass-distilled water was added to the untreated microsomal preparations.

Enzyme assays

All assays were performed at $30 \pm 0.1^\circ\text{C}$ for 6 min with shaking under rigorously controlled conditions. Detergent-treated and intact microsomal preparations were used in each experiment. The production of P_i from Glc-6-P phosphohydrolase was studied by the method of Burchell et al. [29], a modification of the Ames technique [30]. Reaction mixtures (pH 7.0) contained, in 0.3 ml, 40 mM Hepes buffer and 1.0, 1.2, 1.4, 1.8, 2.5, 4.0 or 10 mM Glc-6-P and 10–25 μg of microsomal protein. Standard curves of 0–50 nmol P_i were prepared with each experiment.

The formation of Glc-6-P from carbamoyl-P and Glc by the phosphotransferase reaction was determined by applying a modification [27] of the procedure developed by Noltmann et al. [31]. Reaction mixtures contained 180 mM Glc and 1.0, 1.4, 1.8, 2.5, 4.0 or 10 mM carbamoyl-P. All reaction mixtures (pH 6.5) contained 40 mM sodium cacodylate buffer and 42–65 μg of microsomal protein.

The Fiske-SubbaRow method of P_i determination was employed to study the hydrolysis of PP_i [32]. Reaction mixture (0.6 ml; pH 6.5) contained 40 mM sodium cacodylate buffer, 1.0, 1.4, 1.8, 2.5, 4.0 or 10 mM PP_i and 41–66 μg of microsomal protein. No difference occurred when the protein was removed after termination of the incubation (results not shown). Therefore, the protein was not removed before the colorimetric determination of P_i content. Standard curves of 0–150 nmol of P_i were prepared for each experiment.

Protein was determined by the Pierce bicinchoninic acid method [33], due to its sensitivity and compatibility with the reagents used. The intactness of all microsomal preparations was determined as previously described [34]. Enzyme-activity values

for intact microsomes were calculated (i.e. adjusted for contaminating 'disrupted component') as described by Arion et al. [34]. K_m and $V_{\max}$ values were determined with each set of kinetic data obtained with pooled microsomes from two mice, with the use of the 'Enzfitter' non-linear-regression analysis program [35]. Because carbamoyl-P:glucose phosphotransferase involves a two-substrate system, and kinetic analysis was simplified by keeping glucose concentration high and constant while varying that of carbamoyl-P, we use the primed designations $K'_{\text{carbamoyl-P}}$ and $V'_{\max}$ phosphotransferase [36]. Because kinetic constants determined with intact microsomes apply for the system rather than just the catalytic unit (which is the case with disrupted microsomes), we employ the prefix 'apparent' to all kinetic parameters evaluated with these preparations.

Data presented in Tables 1–3 are mean $\pm$ S.E.M. values which were calculated from individual values obtained with four or more preparations of microsomes [37]. Mean values for the different experimental groups were compared by analysis of variance ('ANOVA' program) [37]; a significant difference was considered to occur when $P < 0.05$.

Latency is defined as the percentage of the total activity determined with a disrupted microsomal preparation that is not manifest in the intact microsomal preparation. Latency was determined at $V_{\max}$ or $V'_{\max}$ by utilizing the equation [19]:

$$\% \text{ latency} = 100 \times (V_d - V_i) / V_d$$

where V_d = mean $V_{\max}$ or $V'_{\max}$ of the disrupted microsomal preparation, and V_i = mean $V_{\max}$ or $V'_{\max}$ of the intact microsomal preparation.

Immunochemical assays

Antibodies to GLUT7 (translocase T_3), translocase T_2 , stabilizing protein and glucose-6-phosphatase (catalytic unit) were as previously described [14,15,38,39]. In control experiments, antibodies to bilirubin UDP-glucuronosyltransferase, androsterone UDP-glucuronosyltransferase, 1-naphthol UDP-glucuronosyltransferase, alkaline phosphatase and GLUT 2 were also used. Protein (10 μg) from each tissue sample was applied to nitrocellulose by using a Bio-Rad dot-blot apparatus. Immunodot analysis was performed with the appropriate antibody, a biotinylated secondary antibody [40] and an ECL detection system [41]. Activated blots were made visible by exposure to Kodak X-OMAT film for 90 s. The films were then scanned with a Joyce-Loebl laser densitometer. The data were interpreted by a Bioscan program for an Apple Macintosh LC computer. The values obtained for control liver proteins were taken as 1, and the values for the other proteins were expressed as a proportion of 1.

RESULTS AND DISCUSSION

Immunochemical studies

Antibodies to the catalytic unit, stabilizing protein and translocases T_2 and T_3 [14,15,38,39] of microsomal glucose-6-phosphatase make it possible to detect these proteins. The studies were performed with liver microsomes from control and tumour-bearing mice; mouse lung microsomes were used as a negative control, as they do not contain several components of the glucose-6-phosphatase system [6]. The negative control was used to demonstrate the background produced by the ECL detection system; in addition, there was minor cross-reactivity of T_3 antibodies to lung glucose-transport proteins (Figure 1). The quantity of translocase T_2 from tumour-bearing microsomes was 2.5 times that of control liver microsomes (Figure 1). The levels

Figure 1 Increase in the T₂ protein of hepatic microsomal glucose-6-phosphatase from tumour-bearing mice compared with control mice

GLUT7 (translocase T₃), SP (stabilizing protein), T₂ (translocase T₂) and G-6-Pase (glucose-6-phosphatase enzyme, EC 3.1.3.9) represent the indicated protein.

of other glucose-6-phosphatase proteins that we measured were unchanged. No probe for translocase T₁ is available to date. Additional studies (results not shown) demonstrated that the levels of several other liver proteins, including microsomal UDP-glucuronosyltransferase isoenzymes (see the Materials and methods section), were also unchanged in tumour-bearing mice. The dramatic increase in the amount of translocase T₂ present in tumour-bearing mice does not in itself indicate a concomitant increase in its transport capacity. Therefore we needed also to determine kinetically if any changes were present in the components of the glucose-6-phosphatase system.

Kinetic studies

The complexity of the glucose-6-phosphatase system means that a complicated kinetic analysis is needed to postulate the effects that a change in the level of any one of the proteins will have on glucose-6-phosphatase activity. The kinetic approach outlined in detail in [7,18] has previously been successfully used to rationalize the activity changes that occur in experimental diabetes [22] and in the genetic deficiencies of the individual proteins (e.g. [9,10,26]). We therefore used the same approach to predict the kinetic changes that would be expected to occur if the increase in T₂ protein in microsomes isolated from the livers of tumour-bearing

mice was accompanied by a concomitant increase in T₂ transport capacity. An increase in T₂ capacity would increase the transport of the product P_i from the microsomal lumen, and would also increase the capacity to transport the substrates carbamoyl-P and/or PP_i into the microsomal lumen.

The predicted consequences of these changes include the following alterations in the kinetic characteristics of the glucose-6-phosphatase system in tumour-bearing mice:

(a) no change in K_m or K'_m and V_{max} , or V'_{max} , values for all activities of disrupted microsomes;

(b) an increase in apparent V_{max} , and K_m for Glc-6-P phosphohydrolase activity in intact microsomes;

(c) a decrease in percentage latency of Glc-6-P phosphohydrolase at V_{max} ;

(d) a decrease in apparent K'_m for carbamoyl-P in the carbamoyl-P:glucose phosphotransferase reaction in intact microsomes;

(e) a possible increase in V'_{max} , for carbamoyl-P:glucose phosphotransferase in intact microsomes;

(f) an increase in V_{max} , for inorganic pyrophosphatase activity in intact microsomes, with accompanying decreased latency based thereon.

Kinetic analysis of the glucose-6-phosphatase system was carried out with microsomes derived from livers of control and tumour-bearing mice to determine if the predicted kinetic changes occurred in the tumour-bearing mice. K_m for Glc-6-P, V_{max} , for Glc-6-P phosphohydrolase and V'_{max} , for phosphotransferase determined with disrupted microsomes from tumour-bearing mice were unchanged from control values (Table 1) and K'_m for carbamoyl-P was slightly increased (Table 2). The absence of change in V_{max} , for Glc-6-P phosphohydrolase and V'_{max} , for phosphotransferase in disrupted microsomes from tumour-bearing mice is consistent with the lack of change in catalytic-unit protein (Figure 1). This contrasts with the response in diabetes, where V_{max} , or V'_{max} , values determined with disrupted microsomes [18–22], as well as catalytic-unit protein [23], increase 3–4-fold. The predicted increase in apparent K_m for Glc-6-P in intact microsomes from tumour-bearing mice was observed, as was an increase in V_{max} , for glucose-6-phosphatase (Table 1). The apparent K'_m for carbamoyl-P in phosphotransferase activity of intact microsomes from tumour-bearing mice decreased, as predicted from the model (Table 2). The V'_{max} , for carbamoyl-P:glucose phosphotransferase activity of intact microsomes from tumour-bearing mice was unchanged (Table 2). The percentage latency value determined at V_{max} , decreased in preparations from tumour-bearing mice with Glc-6-P phosphohydrolase, but latency was unchanged with the carbamoyl-P:glucose phosphotransferase (Table 3). The percentage latency value based on V_{max} , for inorganic pyrophosphatase activity of glucose-6-

Table 1 K_m and V_{max} values for hepatic microsomal glucose-6-phosphatase, with Glc-6-P as substrate from tumour-bearing and control mice

All values are means $\pm$ S.E.M.: * $P < 0.05$ for tumour-bearing versus control group; ** $P < 0.05$ for disrupted microsomes versus intact microsomes.

Experimental group	Intact microsomes		Disrupted microsomes	
	K_m for Glc-6-P (mM)	V_{max} (μ mol/min per mg)	K_m for Glc-6-P (mM)	V_{max} (μ mol/min per mg)
Control	4.0 ± 0.19 ($n = 17$)	0.29 ± 0.02 ($n = 17$)	$1.2 \pm 0.02^{**}$ ($n = 18$)	$0.55 \pm 0.04^{**}$ ($n = 18$)
Tumour-bearing	$7.0 \pm 0.85^*$ ($n = 18$)	$0.42 \pm 0.03^*$ ($n = 18$)	$1.2 \pm 0.05^{**}$ ($n = 20$)	$0.58 \pm 0.04^{**}$ ($n = 20$)

Table 2 K_m for carbamoyl-*P* and V_{max} values for hepatic microsomal carbamoyl-*P*:Glc phosphotransferase from control and tumour-bearing mice

Mean values $\pm$ S.E.M. are presented: * $P < 0.05$ for tumour-bearing versus control group; ** $P < 0.05$ for disrupted microsomes versus intact microsomes. Abbreviation: CP, carbamoyl-*P*.

Experimental group	Intact microsomes		Disrupted microsomes	
	K_m for CP (mM)	V_{max} (μ mol/min per mg)	K_m for CP (mM)	V_{max} (μ mol/min per mg)
Control	9.5 ± 1.5 ($n = 5$)	0.09 ± 0.005 ($n = 5$)	$0.8 \pm 0.05^{**}$ ($n = 6$)	$0.40 \pm 0.03^{**}$ ($n = 6$)
Tumour-bearing	$3.8 \pm 0.5^*$ ($n = 5$)	0.13 ± 0.02 ($n = 5$)	$1.0 \pm 0.07^{*,**}$ ($n = 11$)	$0.58 \pm 0.06^{**}$ ($n = 11$)

Table 3 Latencies of the glucose-6-phosphatase system

Percentage latency values were calculated as described in the Materials and methods section, and are based on V_{max} (phosphohydrolase) or V'_{max} (phosphotransferase) values determined in individual experiments. PP_i phosphohydrolase data are from supplemental studies. All values are expressed as means $\pm$ S.E.M.: * $P < 0.05$ for tumour-bearing versus control groups.

Activity	Experimental group	n	Latency (%)
Glc-6- <i>P</i> phosphohydrolase	Control	17	47.2 ± 5.3
	Tumour-bearing	18	$27.6 \pm 2.3^*$
PP _i phosphohydrolase	Control	6	48.8 ± 3.1
	Tumour-bearing	5	$10.5 \pm 6.2^*$
Carbamoyl- <i>P</i> :glucose phosphotransferase	Control	5	78.2 ± 2.1
	Tumour-bearing	4	77.8 ± 4.4

phosphatase of intact microsomes derived from tumour-bearing mice decreased (Table 3).

Conclusions

These studies describe for the first time a positive response in amount and transport capacity of the translocase T_2 component of the glucose-6-phosphatase system to a perturbation *in vivo*. Thus, kinetic consequences of this alteration in the glucose-6-phosphatase system are of special interest. There was a good correlation between the observed kinetic properties of the enzyme derived from liver microsomes of control and tumour-bearing mice and those differences predicted based on the assumption that the transport capacity of T_2 is increased in the tumour-bearing mice. Although the apparent K_m for Glc-6-*P* of intact microsomes is increased both in diabetes [42,43] and in tumour-bearing mice (Table 1), the mechanism is quite different, an increase in catalytic-unit protein and activity being involved in the former [11,19,27,43], and an increase in translocase T_2 protein and its underlying transport capacity, in the latter.

The physiological relevance of the noted increase in translocase T_2 protein and transport capacity in tumour-bearing mice is not clear, although it would allow increased flux through the glucose-6-phosphatase system. Enhanced Glc-6-*P* levels have been observed in livers of tumour-bearing rats [44]. One possibility is that the increase in apparent K_m for Glc-6-*P* as a result of increased T_2 function observed here could serve to assure this conservation of adequate hepatic Glc-6-*P* in the presence of an enhanced flux [24,25] through the glucose-6-phosphatase system. In turn, this would assure continued function of critical hepatic

metabolic pathways, e.g. the pentose phosphate pathway, under these stressed conditions.

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REFERENCES

- Nordlie, R. C. (1982) in *Membranes and Transport* (Martonosi, A. N., ed.), vol. 1, pp. 263–268, Plenum Publishing Corp., New York
- Nordlie, R. C. (1985) *Trends Biochem. Sci.* **10**, 70–75
- Nordlie, R. C. (1974) *Curr. Top. Cell. Regul.* **8**, 33–117
- Burchell, A. (1990) *FASEB J.* **4**, 70–75
- Nordlie, R. C. and Sukalski, K. A. (1985) in *The Enzymes of Biological Membranes* (Martonosi, A. N., ed.), vol. 2, pp. 349–398, Plenum Press, New York
- Burchell, A. and Waddell, I. D. (1991) *Biochim. Biophys. Acta* **1092**, 129–137
- Arion, W. J., Wallin, B. K., Lange, A. J. and Ballas, L. M. (1975) *Mol. Cell. Biochem.* **6**, 75–83
- Nordlie, R. C. and Sukalski, K. A. (1986) *Trends Biochem. Sci.* **11**, 85–88
- Nordlie, R. C., Sukalski, K. A., Munoz, J. M. and Baldwin, J. J. (1983) *J. Biol. Chem.* **258**, 9739–9744
- Burchell, A. and Waddell, I. D. (1990) *J. Inherited Metab. Dis.* **13**, 247–249
- Burchell, A. (1992) *BioEssays* **14**, 395–400
- Chen, Y. T. and Burchell, A. (1993) in *The Metabolic Basis of Inherited Diseases* (Scriver, C. R., Beaudet, A. L., Sly, W. S. and Valle, D., eds.), 7th edn., McGraw-Hill, New York, in the press
- Waddell, I. D. and Burchell, A. (1991) *Biochem. J.* **275**, 133–137
- Waddell, I. D., Lindsay, J. G. and Burchell, A. (1988) *FEBS Lett.* **229**, 179–182
- Waddell, I. D., Scott, H., Grant, A. and Burchell, A. (1991) *Biochem. J.* **275**, 363–367
- Waddell, I. D., Zomerschoe, A. G., Voice, M. W. and Burchell, A. (1992) *Biochem. J.* **285**, 173–177
- Fulceri, R., Bellomo, G., Gamberucci, A., Scott, H. M., Burchell, A. and Benedetti, A. (1992) *Biochem. J.* **286**, 813–817
- Arion, W. J., Lange, A. J., Walls, H. E. and Ballas, L. M. (1980) *J. Biol. Chem.* **255**, 10396–10406
- Nordlie, R. C. and Snoko, R. D. (1967) *Biochim. Biophys. Acta* **148**, 222–232
- Arion, W. J. and Nordlie, R. C. (1967) *J. Biol. Chem.* **242**, 2207–2210
- Arion, W. J. and Nordlie, R. C. (1965) *Biochem. Biophys. Res. Commun.* **20**, 606–610
- Arion, W. J., Lange, A. J. and Ballas, L. M. (1976) *J. Biol. Chem.* **251**, 6784–6790
- Burchell, A. and Cain, D. I. (1985) *Diabetologia* **28**, 852–856
- Shapot, V. S. and Blinov, V. A. (1974) *Cancer Res.* **34**, 1827–1832
- Singh, J., Grigor, M. R. and Thompson, M. P. (1980) *Cancer Res.* **40**, 1699–1706
- Nordlie, R. C., Scott, H. M., Waddell, I. D., Hume, R. and Burchell, A. (1992) *Biochem. J.* **281**, 859–863
- Nordlie, R. C. and Arion, W. J. (1966) *Methods Enzymol.* **9**, 619–625
- Foster, J. D., Nelson, K. L., Sukalski, K. A., Lucius, R. W. and Nordlie, R. C. (1991) *Biochim. Biophys. Acta* **1118**, 91–96
- Burchell, A., Hume, R. and Burchell, B. (1988) *Clith. Chim. Acta* **173**, 183–192

-
- 30 Ames, B. N. (1966) *Methods Enzymol.* **8**, 115–118
- 31 Noltmann, E., Gubler, C. J. and Kuby, S. A. (1961) *J. Biol. Chem.* **236**, 1225–1230
- 32 Fiske, C. H. and SubbaRow, Y. (1925) *J. Biol. Chem.* **66**, 375–400
- 33 Smith, P. K., Krohn, R. I., Hermanson, G. T., Mallia, A. K., Gartner, F. H., Provenzano, M. D., Fujimoto, E. K., Goeke, N. M., Olson, B. J. and Klank, D. C. (1985) *Anal. Biochem.* **150**, 76–85
- 34 Arion, W. J., Ballas, L. M., Lange, A. J. and Wallin, B. N. (1976) *J. Biol. Chem.* **251**, 4901–4907
- 35 Leatherbarrow, R. J. (1987) *Enzfitter: A Non-linear Regression Data Analysis Program for the IBM PC*, Elsevier-Biosoft, London
- 36 Nordlie, R. C. (1982) *Methods Enzymol.* **87**, 319–353
- 37 Abacus Concepts (1987) *StatView II*, Abacus Concepts, Inc., Berkeley, CA
- 38 Countaway, J. L., Waddell, I. D., Burchell, A. and Arion, W. J. (1988) *J. Biol. Chem.* **263**, 2673–2678
- 39 Burchell, A., Burchell, B., Monaco, M., Walls, H. E. and Arion, W. J. (1985) *Biochem. J.* **230**, 489–495
- 40 Domin, B. A., Serbjit-Singh, C. J. and Philpot, R. M. (1984) *Anal. Biochem.* **136**, 390–396
- 41 Rahi, V. and Stern, P. L. (1990) *Life Sci. (Amersham)* **2**, 9–10
- 42 Segal, H. L. and Washko, M. E. (1959) *J. Biol. Chem.* **234**, 1937–1941
- 43 Hanson, T. L. and Nordlie, R. C. (1970) *Biochim. Biophys. Acta* **198**, 66–75
- 44 Argiles, J. M. and Lopez-Soviano, F. J. (1991) *J. Biol. Chem.* **266**, 2978–2982
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