THE ACTION PATTERN OF THE PHOSPHORYLASE–GLYCOGEN COMPLEX FROM RABBIT SKELETAL MUSCLE†

D. PALM, A. STARKE* and E. HELMREICH
Department of Physiological Chemistry, The University of Würzburg, Medical School, 8700 Würzburg, Germany

Received 28 April 1973

1. Introduction

Action patterns give the number of glycosyl units that are transferred by a glycosyl transferase to and from a polysaccharide. Three mechanisms are generally considered [1–3]: i) Glucose residues are added to or removed from one chain successively. ii) Glucose residues are transferred randomly to or from all available chains and iii) more than one glucose residue is removed or added randomly at each step to all available chains (multirepetitive attack). This is represented in the scheme (fig. 1). Transfer at random (i.e. ii and iii) may be treated according to Poisson's distribution law. In the equation used by Parodi et al. [3] C_+ is the number of chains which have participated in transfer. C_t remains constant and is the number of available chains and N is the total number of glucose residues added or removed: $C_+ = C_t(1-e^{-(N/C_t)})$; for mechanism iii this equation becomes: $C_+ = C_t(1-e^{-(N/C_t \cdot n)})$; where n is the number of residues transferred at each step.

Rabbit skeletal muscle phosphorylase (EC 2.4.1.1; α-1-4-glucan: orthophosphate glucosyltransferase) catalyzes the reaction:

$$\text{Glycogen}_{(n)} + \text{P}_{\text{i}(x)} \rightleftharpoons \text{Glycogen}_{(n-x)} + \text{Glucose-1-P}_{(x)}$$

Parodi et al. [3] found an average of 1.2 to 1.4 glycosyl units per step transferred by phosphorylase *b*. The action pattern did not appear to be much influenced by the glycogen preparations, which were used in that work. We have studied independently action patterns with crystalline phosphorylases *b* and *a*, and different glycogen preparations but mainly in the physiological direction of glycogen breakdown and have obtained values of n ranging from 1.2 to 1.6 (G. Eckert and D. Palm, unpublished experiments, 1970 [4]). Since the earlier experiments gave similar action patterns in the direction of synthesis and degradation mainly glycogen synthesis was followed in the present work. In order to see whether the deviation from 1 (or any integral number) might reflect variations in the reac-

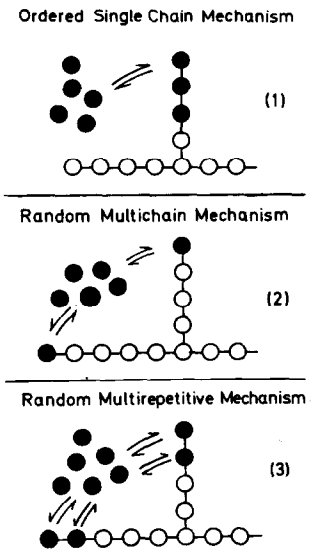

Fig. 1. Possible mechanisms of glycosyl transfer.

† This paper is dedicated to Professor Theodor Wieland on the occasion of his 60th birthday.

* This work is part of the M.D. thesis of A.S., University of Würzburg, 1973.

tivity among glycogen chains, experiments with the protein glycogen complex, prepared from rabbit muscle according to Meyer et al. [5] were carried out. It was assumed that in this particle which closely resembles the native state the primer specificity of available chains may be more uniform as is the case in the enzyme–substrate complex formed from its isolated components *in vitro*. The results have confirmed this view.

2. Materials and methods

Phosphorylase *b* was prepared from fresh rabbit skeletal muscle [6]. It was recrystallized at least three times. Phosphorylase *a* was prepared from phosphorylase *b* with phosphorylase *b* kinase, ATP and Mg^{2+} and separated from the other proteins by repeated crystallization [7]. The protein–glycogen particle was prepared from rabbit skeletal muscle [5]. The preparation was used fresh to minimize amylase action. Glycogen from rabbit liver (grade I, Boehringer) was free of 5′-AMP. The nonreducing terminal glycosyl residues were determined with periodate and found to be 5.73 ± 0.38% [8]. The molecular weight distribution was determined by sucrose (10–40%) density gradient centrifugation according to Mordoh et al. [9] (see fig. 2). Glycogen concentrations were determined by dry weight [11]. They are expressed in terms of nonreducing terminal glycosyl residues: (1 g/100 ml = 3.5 × 10^{-3} M). Glc-1-P-Na, ATP and other substrates were purchased from Boehringer. [U-^{14}C]D-Glc-1-P-K, 233 mCi per mmol, methyl [U-^{14}C]α-D-glucopyranoside, 3 mCi per mmol, were obtained from the Radiochemical Centre, Amersham. All other reagents were of the purest available grade.

Phosphorylase activity was measured in the direction of glycogen synthesis in the presence of Glc-1-P. P_i liberated was analyzed according to Fiske and Subbarow [12] using an automated procedure [13]. Protein concentrations were determined using $E^{1\%cm^{-1}}$ = 13.2 [14]. Phosphorylase activities in the protein glycogen complex were measured by the incorporation of [U-^{14}C]Glc-1-P into glycogen [15].

For determining action patterns the reaction was carried out except as stated otherwise, at 31°C and pH 6.8 in 5 ml of a solution of 10 mM glycerol-2-P, 2 mM EDTA, 1 mM 2-mercaptoethanol, 2.2 mM 5′-AMP, containing 10 mg glycogen and 5 μCi = 21.5

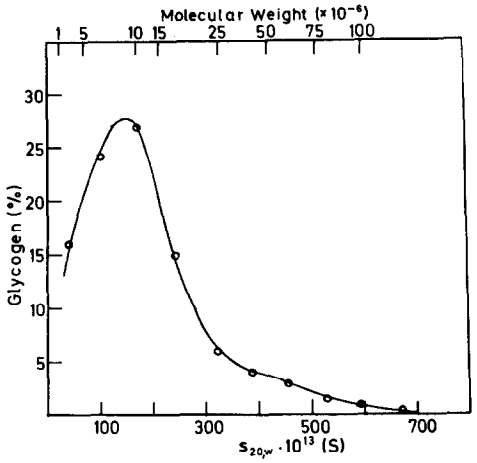

Fig. 2. Molecular weight distribution of rabbit liver glycogen (Boehringer, grade I). Sedimentation coefficients were determined in a SW 50.1 rotor at 20 000 rpm from 30 to 120 min at 0°C; $s_{20,w}$ values were calculated according to McEwen [10]. Approximate molecular weights (g·mol^{-1}) were calculated as described by Mordoh et al. [9]. On the ordinate glycogen is expressed in % of total added.

nmoles [U-^{14}C]Glc-1-P. Phosphorylase *b* and *a* concentrations ranged from 2.2–11 and from 0.5–1 μg per ml (10^{-8} M) respectively. With phosphorylase *a*, AMP was omitted. In the linear range of the rate curves up to 6 samples were withdrawn at different time points. Each experiment was run in duplicate or triplicate. The reaction was stopped by heating the samples for 2 min in a boiling water bath. Glycogen was isolated from the reaction mixture by addition of 1.1 times the volume of anhydrous ethanol and centrifugation in the cold. The glycogen pellet was redissolved in 2 ml H_2O, reprecipitated and washed repeatedly with 60% (v/v) ethanol to constant specific radioactivity with a total recovery of 91–99%. Glycogen samples dried in a desiccator, were carefully weighed for the preparation of 2% stock solutions. Aliquots, 0.05 ml and 0.1 ml respectively, were counted and treated with $NaIO_4$. A suspension of the protein glycogen complex (0.2 ml) containing about 2 mg of endogenous glycogen was supplemented by addition of substrates and salts to a final concentration of 2.1 mM $CaCl_2$, 10 mM Mg acetate and 0.5–6 μCi, i.e. 2.2–25.8 nmoles [U-^{14}C]Glc-1-P, pH 6.8, and to a total volume of 0.23–0.26 ml. The reaction was started with 10 μl of a 0.1 M ATP solution. After 30 to 40 sec at 30°C the reaction was stopped with 0.2

ml M $HClO_4$. The reaction mixture was neutralized with K_2CO_3, and the denatured protein and $KClO_4$ were removed by centrifugation in the cold. Glycogen was isolated from the supernatant as described. The extent of the reaction was set by the Glc-1-P concentration. The specific radioactivity and the concentration of Glc-1-P in each sample were determined making allowance for endogenous Glc-1-P in the particle suspension (usually about 3×10^{-5} M, as determined spectrophotometrically in a coupled enzyme assay [16]).

The terminal, nonreducing glycosyl residues were determined as described [3,8]. Radioactive formic acid was separated from the other radioactive material by paper electrophoresis as described by Parodi et al. [3] or by chromatography on Sephadex G-25. The formic acid peak was cut out and counted either in a toluene:Triton X-100 1:1 (v/v) mixture or in "Instagel" (Packard Co.) in a Packard Model 3380 liquid scintillation spectrometer. The usual corrections for quenching, background and efficiency were applied. The percent incorporation of terminal glycosyl residues was calculated by multiplying the radioactivity of the formic acid (cpm $\times$ μmoles^{-1}) with the factor 6 and dividing it through the total radioactivity of the glycogen sample (cpm $\times$ μmole^{-1}). As a control, methyl-[U-^{14}C]α-D-glucopyranoside was treated with periodate and the reaction product subjected to electrophoresis under the same conditions.

The distribution of the incorporated [^{14}C]glycosyl residues in glycogen was determined according to [3] (see also Introduction). Assuming random incorporation, the probability of all transfer steps is given by the Poisson distribution. In this case and when N is small, the number, n, of glycosyl residues incorporated in each step may be calculated from the expression: $n = N/C_+$. Thus when the extent of incorporation is very small as was the case in our experiments ($N \leqq 1\%$ of all terminal glycosyl residues in glycogen), the extent of chain elongation can be calculated from the fraction of labeled terminal glycosyl residues. Calculations were carried out with a Wang 720 B calculator. For further details and the method of graphic extrapolation used for the determination of n, the original paper of Parodi et al. [3] may be consulted.

For the preparation of labeled glycogen for degradation studies, 50 mg glycogen, 50–500 μmol Glc-1-P and 20 μCi [U-^{14}C]Glc-1-P were incubated in 10 ml of 50 mM glycerol-2-P, 2 mM EDTA and 2.5 mM 5′-AMP buffer, pH 6.8 with phosphorylase b. The reaction was terminated after incorporation of less than 1 glucose unit per nonreducing terminal glycosyl residues.

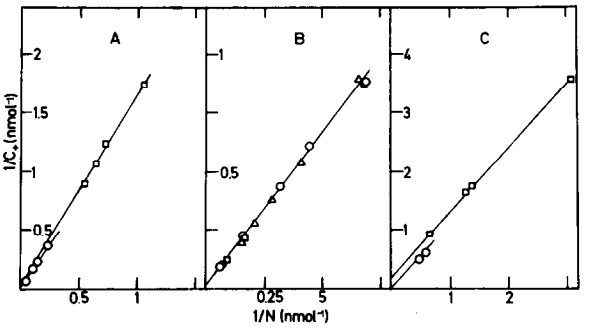

Fig. 3. Experimental determination of action patterns from the ratio of incorporation of [^{14}C]glucose into terminal positions during glycogen synthesis catalyzed by (A) phosphorylase b; (B) phosphorylase a; and the (C) glycogen–protein complex. The reciprocal of total uptake ($1/N$) and the fraction incorporated into the terminal residues ($1/C_+$) are given on the abscissa and ordinate, respectively. □, ○, △ Data from several independent series of experiments. The action patterns correspond to the slopes of the regression lines. The ordinate intercepts in C indicate that the glycogen chains in the complex are available to phosphorylase to a variable degree. The slopes are the same.

In one case nonradioactive Glc-1-P was added in large excess in order to elongate the chains before incorporation of glucose from labeled Glc-1-P. Degradation to varying degree was studied in the same reaction mixture but replacing Glc-1-P by 20 mM P_i. In this case, the reaction was followed by assaying the Glc-1-P formed [16].

3. Results

The results are presented in fig. 3 and table 1. From the slopes in fig. 3 the values for the pattern "n" of synthesis were calculated. They are given together with the mean elongation of chains (N/C_+) in table 1. As may be seen the lower limit of N/C_+ closely coincides with n.

Action patterns for glycogen degradation may be effected by the labeling pattern of the glycogen since the glycosyl donor which is subsequently degraded was synthesized by the same enzyme. This actually is

Table 1
Action patterns of phosphorylase catalyzed glycosyl transfer.

	N (nmoles glucose added or removed per mg glycogen)	C_+/N (Fraction of terminal [^{14}C]glucose)	N/C_+ (Average chain elongation)	n*
Synthesis by phosphorylase *b*	0.094 – 0.185	0.61 – 0.59	1.63 – 1.69	1.61
	0.415 – 2.34	0.56 – 0.61	1.55 – 1.65	1.55
Synthesis by phosphorylase *a*	0.654 – 1.50	0.69 – 0.64	1.44 – 1.57	1.36
	0.076 – 1.59	0.78 – 0.62	1.28 – 1.60	1.35
	0.152 – 0.65	0.76 – 0.73	1.32 – 1.36	1.37
Synthesis by the phosphorylase protein–glycogen complex	0.18 – 0.78	1.0 – 0.64	1.0 – 1.56	1.0
	0.95 – 1.09	0.93 – 0.92	1.07 – 1.09	1.09
	N	Fraction of terminal [^{14}C]glucose	(nmoles [^{14}C]glucose removed per mg glycogen)	n**
Degradation by phosphorylase *b*	185	0.66	85	1.38
	202		81	1.71
	267		98	1.64
	386		114	1.74

* The action pattern was calculated from the slopes of fig. 1, ($1/C_+ = n/N$); ** n was calculated from $C_+ = C_t(1-e^{-N/C_t \cdot n})$ according to mechanism 3. The average of the four recorded degradation experiments was $n = 1.62 \pm 0.16$. The value of n would be reduced to 1.12 ± 0.12 if one assumes that the labeled residues remaining after exhaustive degradation (17%) were originally non-terminal (see Results).

suggested by the analysis of the labeled glycogen sample before and after exhaustive degradation. The fraction of labeled terminal ends measured with periodate was 0.81. After exhaustive degradation by phosphorylase 17% of the label could not be removed, however the remaining labeled residues were all accessible to periodate. This demonstrates that some glucosyl residues added prior to the glycogen by phosphorylase *in vitro*, were not degraded subsequently on incubation with the same enzyme. For calculation of the experimental degradation patterns we have taken this distribution of labeled residues into account. Furthermore, we have assumed with Parodi et al. [3] that 41% of all terminal residues are available (i.e. $C_t = 41\%$). This value corresponds to the specific radioactivity of Glc-1-P incorporated into glycogen during synthesis.

4. Discussion

An action pattern as usually defined gives only a statistically reproducible value of several events occurring simultaneously or successively. Among others, the numerical value reflects the specificity of the enzyme for the acceptor–donor chain. The steric complementarity depends on the structure of the macromolecular substrate which determines the likelihood of encounter and the lifetime of the enzyme–substrate complex. Although these problems have been recognized [3, 17, 18], it is not known to what extent these factors are responsible for the deviation of an action pattern from an integral number. Our results are in accordance with those of Parodi et al. [3]: On the basis of this evidence it seems that crystalline muscle phosphorylase preparations catalyze the transfer of more than one glycosyl unit per step to glycogen as acceptor. In our experiments, the acceptor was a commercial rabbit liver glycogen preparation (cf.

fig. 1). We have considered the possibility that multiple attack by phosphorylase might reflect differences in primer specificity. If this were the case, a probability treatment, such as Poisson's distribution could give erroneous results because it only distinguishes in all or none fashion between available and nonavailable chains, but neglects gradual differences in primer specificity. We have tried to minimize these difficulties by using highly labeled [U-^{14}C]Glc-1-P which allowed to restrict the extent of the reaction. Whelan and Bailey [1] and Illingworth et al. [17] have suggested that when the turnover is small, the enzyme will use preferentially the primer with the best fit. We thus interpret action patterns as high as 1.61 to indicate chain elongation of those primer molecules best fitted to the enzyme (table 1).

However, whenever the action pattern is > 1.0, one cannot decide whether chain elongation is catalyzed by a single active center, transferring more than one glycosyl unit per step or by the action of several sites all acting on the same chain, but each site transferring only one unit at a time (cf [2]). In order to overcome this ambiguity, experiments with the protein glycogen –complex of rabbit muscle were undertaken. It was assumed that in the particle each phosphorylase molecule is bound more or less irreversibly to a glycogen chain and that all the glycogen chains are rather uniform with respect to chain length. If this should be the case one would not expect the glycogen chains to grow disproportionately when a pulse of highly labeled Glc-1-P is applied. Under these conditions an action pattern of 1 was obtained, which is compatible with these assumptions (see table 1, fig. 3). This then indicates that each phosphorylase molecule, when it is integrated in a stoichiometric complex with glycogen molecules of equal chain lengths only transfers one glycosyl residue at a time to its acceptor chain. This probably relates more closely to the living state than the enzyme–substrate complex formed in the test tube.

Acknowledgements

Supported by research grants of the Deutsche Forschungsgemeinschaft, the Federal Ministry of Education and Science and the "Fonds der Chemie".

References

[1] W.J. Whelan and J.M. Bailey, Biochem. J. 58 (1954) 560.
[2] D. French and G.M. Wild, J. Am. Chem. Soc. 75 (1953) 4490.
[3] A.J. Parodi, J. Mordoh, C.R. Krisman and L.F. Leloir, European J. Biochem. 16 (1970) 499.
[4] Work carried out in 1970 by G. Eckert as partial requirement for the M.D. Thesis to be submitted to the Medical Faculty, University of Würzburg.
[5] F. Meyer, L.M.G. Heilmeyer, Jr., R.H. Haschke and E.H. Fischer, J. Biol. Chem. 245 (1970) 6642.
[6] E.H. Fischer and E.G. Krebs, J. Biol. Chem. 231 (1958) 65.
[7] R.J. De Lange, R.G. Kemp, R.D. Riley, R.H. Cooper and E.G. Krebs, J. Biol. Chem. 243 (1968) 2200.
[8] F.W. Fales, Anal. Chem. 31 (1959) 1898.
[9] J. Mordoh, C.R. Krisman and L.F. Leloir, Arch. Biochem. Biophys. 113 (1966) 265.
[10] C.R. McEwen, Anal. Biochem. 20 (1967) 114.
[11] M. Dubois, K.A. Gilles, J.K. Hamilton, P.A. Rebers and F. Smith, Anal. Chem. 28 (1956) 350.
[12] C.H. Fiske and Y. Subbarow, J. Biol. Chem. 66 (1925) 375.
[13] R.H. Haschke and L.M.G. Heilmeyer, Jr., Anal. Biochem. 47 (1972) 451.
[14] L.L. Kastenschmidt, J. Kastenschmidt and E. Helmreich, Biochemistry 7 (1968) 3590.
[15] J.A. Thomas, K.K. Schlender and J. Larner, Anal. Biochem. 25 (1968) 486.
[16] H.U. Bergmeyer and G. Michal, in: Methoden der enzymatischen Analyse, 2nd edition, ed. H.U. Bergmeyer (Verlag Chemie, Weinheim, 1970) p. 1196.
[17] B. Illingworth, D.H. Brown and C.F. Cori in: Ciba foundation symposium "control of glycogen metabolism". eds. W.J. Whelan and M.P. Cameron (Churchill, London, 1964) p. 107.
[18] D.H. Brown, B. Illingworth and R. Kornfeld, Biochemistry 4 (1965) 486.