How Did Glycogen Structure Evolve to Satisfy the Requirement for Rapid Mobilization of Glucose? A Problem of Physical Constraints in Structure Building

Ruth Meléndez,[1] Enrique Meléndez-Hevia,[2] Marta Cascante[1]

[1] Universitat de Barcelona, Departament de Bioquimica i Biologia Molecular, Facultat de Quimica, Martí i Franquès 1, 08028 Barcelona, Spain
[2] Universidad de La Laguna, Departamento de Bioquímica, Facultad de Biología, 38206 Tenerife, Canary Islands, Spain

Abstract. Optimization of molecular design in cellular metabolism is a necessary condition for guaranteeing a good structure–function relationship. We have studied this feature in the design of glycogen by means of the mathematical model previously presented that describes glycogen structure and its optimization function [Meléndez-Hevia et al. (1993), Biochem J 295: 477–483]. Our results demonstrate that the structure of cellular glycogen is in good agreement with these principles. Because the stored glucose in glycogen must be ready to be used at any phase of its synthesis or degradation, the full optimization of glycogen structure must also imply the optimization of every intermediate stage in its formation. This case can be viewed as a molecular instance of *the eye problem*, a classical paradigm of natural selection which states that every step in the evolutionary formation of a functional structure must be functional. The glycogen molecule has a highly optimized structure for its metabolic function, but the optimization of the full molecule has meaning and can be understood only by taking into account the optimization of each intermediate stage in its formation.

Key words: Glycogen — Evolution of metabolism — Natural selection — Optimization — Molecular structure — Molecular optimization

Introduction

Evolutionary optimization of metabolism is a field that has been developed in the last 10 years. Chemical design of several pathways have been analyzed under this approach, including the pentose phosphate cycle (Meléndez-Hevia 1990; Meléndez-Hevia and Isidoro 1985; Meléndez-Hevia and Torres 1988; Meléndez-Hevia et al. 1994), Calvin's cycle in photosynthesis (Meléndez-Hevia 1990), the Krebs citric acid cycle (Meléndez-Hevia et al. 1996), and glycolysis (Heinrich et al. 1997; Meléndez-Hevia et al. 1997a). The optimization of kinetic parameters of enzymes has also been demonstrated (Heinrich and Hoffmann 1991; Heinrich et al. 1987, 1991). Genetic algorythms (Gilman and Ross 1995) and the quasi-species model (Montero et al. 1996) have also been demonstrated to be useful as optimization tools to investigate metabolic design. Another interesting subject in this field is the investigation of the structure of molecules involved in metabolism. Here, glycogen is a good example of a highly organized cellular molecule, where a close structure–function relationship has been mathematically proved (Meléndez-Hevia et al. 1993, 1995).

A schematic picture of glycogen is shown in Figure 1. It is formed by chains of glucose polymerized by $1 \rightarrow 4$ glycosidic bonds. Each chain has an average length of 13 glucose residues, and each internal chain has two branching points by $1 \rightarrow 6$ glycosidic bonds generating two new chains. The molecule has a spherical shape, with the chains organized in concentric tiers, and the full mol-
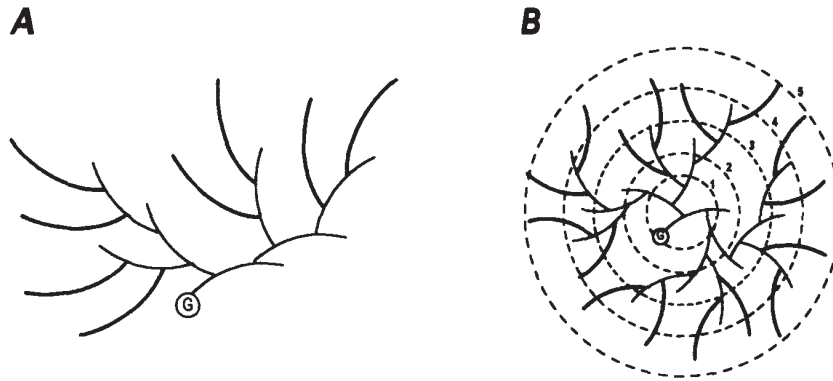
A **B**

Fig. 1. Schematic drawing of the glycogen structure. A schematic view of the glycogen molecule showing the homogeneous structure and its spherical shape formed by concentric tiers (marked by numbered dashed circles). Drawing based on Whelan's model (Gunja-Smith et al. 1970) and other data from Goldsmith et al. (1982); see also Meléndez-Hevia et al. (1993). G, glycogenin, primer protein which promotes the synthesis of the first chain of glycogen from *tyr*-194. All chains of the molecule have the same length (13 glucose residues) and each (**B**) chain (in the inner tiers) has two branching points with two new chains. (**A**) Chains, drawn in bold, are in the most external tier, they are nonbranched, and contain the glucose directly available for phosphorylase, which represents 34.6% of the stored glucose, independently from the number of tiers of the molecule (at any growth state of the molecule). The picture is very simplified, with only five tiers.

ecule of cellular glycogen has 12 tiers. It is generally accepted that size limit is determined by the density of the last tier because the enzymes also occupy space, and when density reaches a certain value they have no room left to attach to the molecule and continue working. Data about size of the enzymes that build the molecule (synthase and branching) are not clear (molecular weight of the synthase monomer is about 85,000, but it is not clear whether the active form is the monomer, the dimer, or the tetrameric form; see, e.g., Westphal and Nuttal 1992). In any case, independently of the quantitative data about it, such a limit must exist since the crowding in the last tier grows exponentially as the number of tiers is increasing, and so leaving no room for the activity of the synthesizing enzymes. This fact was first predicted by Madsen and Cori (1958) and afterwards discussed by Goldsmith et al. (1982) and Meléndez-Hevia et al. (1993). These two last papers contain some calculations about the space available for the enzymes: in the 12th tier glucose occupies 26% of the space; in a hypothetic 13th tier (which obviously would be physically impossible to build) the space occupied by glucose would be 62%.

The optimization of glycogen was first studied by Meléndez-Hevia et al. (1993) by means of mathematical modelling. That model describes the structural properties of the glycogen molecule, according to Whelan's model (see Gunja-Smith et al. 1970 and Goldsmith et al. 1982) with three parameters (independent variables), and four (response) variables, as follows:

Parameters

r = branching degree
t = number of tiers;
 t_{max} = maximal possible number of tiers
g_c = number of glucose residues in a chain

Variables

C_A = number of A chains (external chains)

$$C_A = r^{(t-1)} \tag{1}$$

G_T = total number of glucose residues stored in a molecule of glycogen

$$G_T = g_c \cdot \frac{1 - r^t}{1 - r} \tag{2}$$

G_{PT} = number of residues available to phosphorylase

$$G_{PT} = C_A \cdot (g_c - 4) \tag{3}$$

V_S = total volume of the molecule (assuming that it is a sphere).

$$V_S = \frac{4}{3}\pi \cdot t^3 \cdot (0.12 \, g_c + 0.35)^3 \tag{4}$$

It should be noted that the physical constraints concerning phosphorylase mechanism, and volume of the glucose molecule are considered in Equations (3) and (4). The optimized structure of glycogen was found maximizing the function

$$f_{opt} = \frac{G_T \cdot C_A \cdot G_{PT}}{V_S} \tag{5}$$

whose physical meaning is to store the maximum possible amount of glucose directly available to phosphorylase in the most possible compact (highest density) molecule (Meléndez-Hevia et al. 1995).

Stating the branching degree as $r = 2$, Eq. (5) can be expressed as

$$f_{opt} = K \frac{g_C \cdot (g_C - 4)}{(0.12g_C + 0.35)^3}$$

where K is a function of t_{max}. Thus, the optimization function can be written as

$$f_{opt} = K(t) \cdot F(g_c) \qquad (6)$$

Since t and g_c are independent variables, the value of g_c that maximizes f_{opt} is independent from the value of t considered, it being maximum for $g_c = 13$. This value agrees with the experimental results of chain length obtained by many authors (Table 1). Nevertheless, this describes the optimization of a function which depends only on one independent variable: the chain length g_c. The structure of glycogen depends, however, on more parameters—and particularly on the branching degree r. Thus, the questions now are as follows: (a) Which parameters are really involved to fully optimize the structure of the glycogen molecule? (b) By means of which mechanisms can the cell metabolism control them? (c) How can natural selection account for them?

In this work, we have tried to give an answer to these questions. Our results demonstrate that they relate mainly to physical—rather than chemical—problems of cellular materials such as density and the procedure to organize a set of chemical reactions in such a way that they build a homogeneous and highly efficient material for energetic metabolism. Our results on the optimization function demonstrate that the structure of cellular glycogen can only be explained taking into account the usefulness of each intermediate stage in its formation; this easily reminds us of the classic *eye problem*, and confirms once again the sentence of Dobzhansky (1973) "Nothing in Biology makes sense except in the light of evolution."

Theorical Approach and Results

Mechanism of Glycogen Synthesis

The mechanism of glycogen growth has been precisely determined, as it is shown in Figure 2A (see, e.g., Stryer 1995). There is a minimal possible distance between branches, because the branching enzyme needs certain room to work; this was empirically determined as 4 glucose residues (Walker and Whelan 1960); thus, a glycogen with $r = 2$ needs a chain length of $g_c > 10$; a glycogen with $r = 3$ needs $g_c > 15$, and so on. Therefore, cases with $r = 2$ and $g_c < 11$ or those with $r = 3$ and $g_c < 16$, and so on, are not possible. Figure 2B shows a general view of the process of molecular growth. Glycogen synthase (S) promotes the growth of each chain, and the branching enzyme (B) makes branching points on them when the physical constraints allow it. That

picture is, of course, a simplified view, but it can be considered as statistically representative of how glycogen growth is carried out. The growth of all chains simultaneously can be easily explained assuming that glycogen synthase works statistically on all chains. A statistical activity of the branching enzyme on the surface of the molecule can also be assumed.

The mechanism shown in Figure 2B describes the general features of the glycogen growth, but the fine structure of the molecule is determined by the activity of the two enzymes, as the ratio of activity between them accounts for the chain length and the branching degree (r). Thus, it is important to know which parameters the cell controls to build the glycogen molecule, and how. Two different possibilities can be considered.

Case A. Activity of the Branching Enzyme Limited

In this case there is a certain ratio of activity between the two enzymes; then each branch grows by the activity of synthase, and the branching points are set on it at regular intervals, according the activity of the branching enzyme. This mechanism leads us to an optimization function with two independent variables (g_c and r), which is plotted in Figure 3. There is a curve corresponding to each value of r and the t_{max} permitted in each case, but only some particular zones of each curve have physical meaning: the zones drawn in dashed lines, at the left side of the full symbols are not permitted, because their r values are not possible with those values of g_c (for example, for $r = 2$, the chain length must be $g_c \geqslant 11$). On the other hand, the maximum possible number of tiers (t_{max}) depends on the chain length, because a molecule with longer chains is less dense (see Fig. 4); thus, the right-side values of each curve do not represent real values of f_{opt} since for those g_c one more tier is allowed, and a discontinuity (*vertical arrows* in the Fig. 3) appears changing to the next curve, with a higher value of t_{max}. t_{max} also depends on the branching degree, because a high branching degree produces a highly dense molecule where less tiers are allowed; this effect can also be seen in Figures 3 and 4.

Case B. Excess of the Branching Enzyme Activity

In this case, the branching enzyme has such an activity that a new branch always appears as soon as possible. Then, glycogen synthase promotes the growth of all branches, and the branching enzyme makes a branching point when the chain length allows it. This mechanism produces a glycogen molecule where the branching degree r is always the highest possible value according to its chain length; thus the glycogen molecule has the thickest structure (under the physical constraints mentioned above of the four glucose residues necessary between branches), and there is only one independent variable: g_c, because the chain length determines when the branching enzyme can work. In this case, the optimiza-

Table 1. Chain lengths measured in glucose residues of glycogen from several sources, analysed with different methods.[a] [See also Calder (1991); Ryman and Whelan (1971)].

Source	Chain length (g_c)	Reference
Mammals		
Human liver	15	Bathgate and Manners (1966)
Human muscle	11–12	Manners (1957); Gunja-Smith et al. (1971)
Human placenta	11–12	Blows et al. (1988)
Cat liver	12–14	Illingworth et al. (1952); Manners (1957); Gunja-Smith et al. (1971)
Dog liver	12	Hassid and Chaikoff (1938)
Horse diaphragm	15	Gunja-Smith et al. (1971)
Horse muscle	12	Bell (1944)
Horse liver	11	Abdel-Akher and Smith (1951)
Ox liver	12–13	Abdel-Akher and Smith (1951)
(fetal) Guinea-pig liver	13	Illingworth et al. (1952)
Mouse liver	13–14	Calder (1987); Kjolberg et al. (1963); Calder (1987)
Mouse muscle	13	
Pig muscle	11–16	Kjolberg et al. (1963)
Pig liver	15–16	Kjolberg et al. (1963)
(fetal) pig liver	11	Greenwood and Manners (1957)
Rabbit liver	12–14	Calder (1987); Bullivant et al. (1983); Gunja-Smith et al. (1971)
Rabbit muscle	13–14	Calder (1987); Gunja-Smith et al. (1971); Kjolberg et al. (1963); Manners and Wright (1962)
Rat liver	12–13	Calder and Geddes (1986); Manners and Wright (1962); Illingworth et al. (1952)
Rat muscle	13	Calder and Geddes (1985)
Fetal sheep liver	13	Harrap and Manners (1952); Bell and Manners (1952)
Birds		
Hen liver	15	Kjolberg et al. (1963)
Fish		
Skate	12–13	Gunja-Smith et al. (1971); Liddle and Manners (1957)
Dogfish	12	Haworth et al. (1939)
Bass (*Micropterus dolamieu*)	14	Abdel-Akher and Smith (1951)
Bullhead (*Ameiurus melas*)	12	Abdel-Akher and Smith (1951)
Carp (*Cyprinus carpio*)	12	Abdel-Akher and Smith (1951)
Crappie (*Pomoxis annularis*)	12	Abdel-Akher and Smith (1951)
Garfish (*Lepisosteus osseus*)	12	Abdel-Akher and Smith (1951)
Haddock (*Gadus aeglefinus*)	12	Haworth et al. (1939)
Hake (*Merluccius vulgaris*)	12	Haworth et al. (1939)
Northern pike (*Esox estor*)	12–13	Abdel-Akher and Smith (1951)
Walleyed pike (*Stizostedion vitreum*)	13	Abdel-Akher and Smith (1951)
Mollusca		
Helix pomatia	11–12	Baldwin and Bell (1940)
Polychaeta		
Arenicola	11	Liddle and Manners (1957)
Platyhelminthes		
Moniezi expansa	12	Abdel-Akher and Smith (1951)
Nematoda		
Ascaris lumbricoides	12–15	Gunja-Smith et al. (1971); Bell (1944)
Yeast		
Saccharomyceas cerevisiae	12–13	Northcote (1953); Manners and Maung (1955)
Protozoa		
Trichomonas foetus	14–15	Manners and Ryley (1955); Gunja-Smith et al. (1971)
Tetrahymena pyriformis	13	Manners and Ryley (1952)
Bacteria		
Escherichia coli	11	Boyer and Preiss (1977)
Bacillus megatherium	10–11	Barry et al. (1953)

[a] Lomako et al. (1993) have shown that the molecular weight (MW) of proglycogen and macroglycogen from astrocytes are the same as those from muscle. Although they do not report chain length data, the identity of the MW suggests that glycogens from both sources have the same structure.
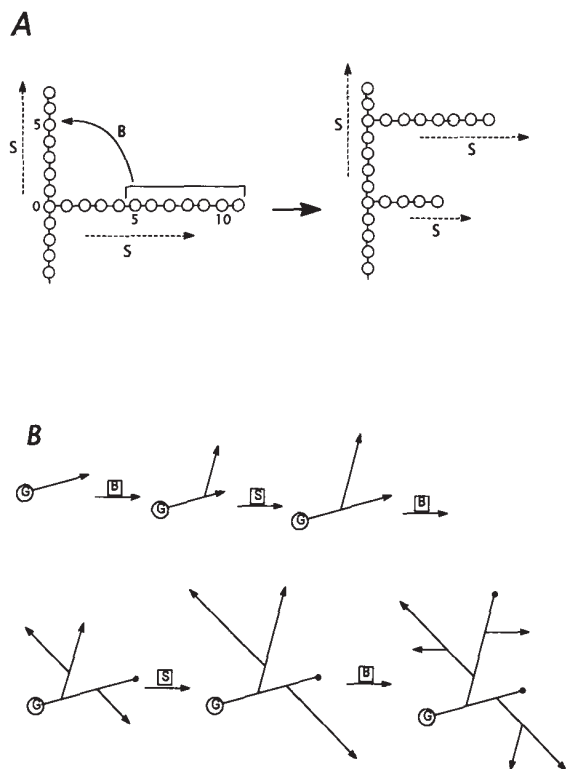
A

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Fig. 2. Mechanism of glycogen synthesis. (A) Detailed mechanism of the branching enzyme. Glycogen synthase (S) promotes the growth of the chains polymerizing glucose units by 1 → 4 bonds, and the branching enzyme (**B**) produces the growth of new chains making 1 → 6 bonds between glucoses. The branching enzyme transfers a block of 7 glucose residues including the nonreducing end from a branch; the new branch point must be at least at the fifth residue from a preexisting one. (B) Pattern of glycogen synthesis. Glycogen synthase (S) produces the growth of all branches until one of them has 13 glucose residues; then the branching enzyme works producing a new branch. Chains growing are denoted by *arrows*, and the ends of the full chains are marked with *black points*. Synthesis of the complete molecule of glycogen from glycogenin occurs in two stages that produce proglycogen (glycogen with 7–8 tiers) and macroglycogen (full glycogen with 12 tiers). The enzymes that operate in each phase are different (Lomako et al. 1993), but their action mechanisms are the same in both cases as is shown in this picture.

tion function f_{opt} becomes discontinuous, with a new discontinuity appearing every 5 residues along the chain, when a new branch can be built on it. The structure corresponding to the parameters ($r = 2$; $g_c = 13$; $t = 12$) is the absolute maximum (see Fig. 5).

It is not difficult to derive which of these two possibilities works in the cell. All experimental data demonstrate that the first possibility must be discarded, and that cell work realizes the second, namely, conditions of excess of the branching enzyme. Data supporting this conclusion are as follows:

1. Activity of both enzymes: The branching enzyme has much higher activity (about 200-fold) than glycogen synthase, according with the results reported by
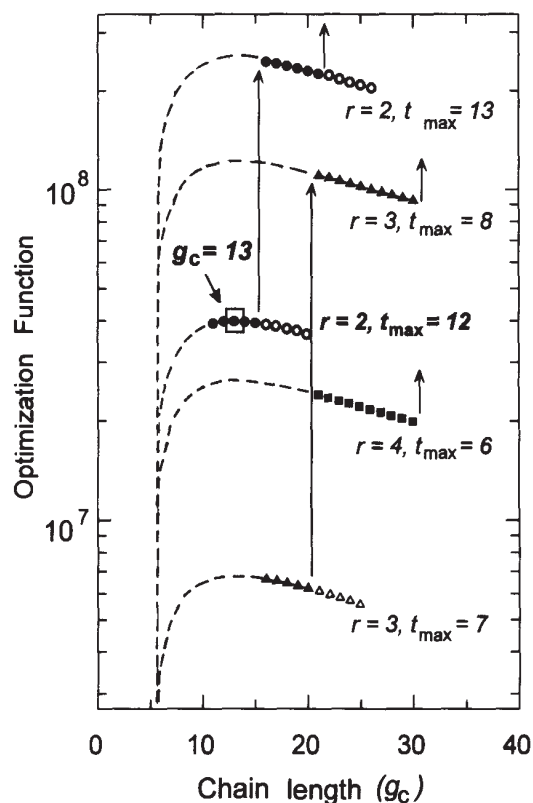

Fig. 3. Optimization functions if glycogen is synthesized under limited activity of the branching enzyme. In this case, the optimization function [$f_{opt} = (G_T \cdot G_{PT} \cdot C_A)/V_S$] has two independent variables (the chain length g_c and the branching degree r); there is a curve for each value of r and t_{max}; only zones of the curves with full symbols have physical meaning. Zones drawn with dashed lines at the left side are not permitted, because their r value is not possible with that value of g_c (for example, for r = 2, the chain length must be $g_c \geq 11$); also the right side values of each curve (open symbols) does not represent real values of f_{opt}, since for those g_c values, one more tier is allowed; this gives rise to the discontinuities represented by vertical arrows, toward the function with one more tier.

Brown and Brown (1966), Camici et al. (1984), Caudwell and Cohen (1980), Golden et al. (1977), Krisman (1962), and Larner (1955).

2. Regulation of glycogen synthesis: Glycogen synthesis seems to be based only on the activity of synthase, which is regulated by covalent and allosteric mechanisms (see a review in Meléndez-Hevia et al. 1997b), and no special mechanism to regulate the activity of the branching enzyme is known to be working in the process. Also, different physiological conditions such as the nutritional state induce changes in the activity of synthase (fasting induces an increase of activity of synthase of about two- to three-fold; see Hue et al. 1975; Stalmans et al. 1974), and no change of activity of branching enzyme has been reported in similar situations. Furthermore, a recent result reported by Skurat et al. (1996) shows that glycogen synthesis can be increased by overexpression of synthase, but not
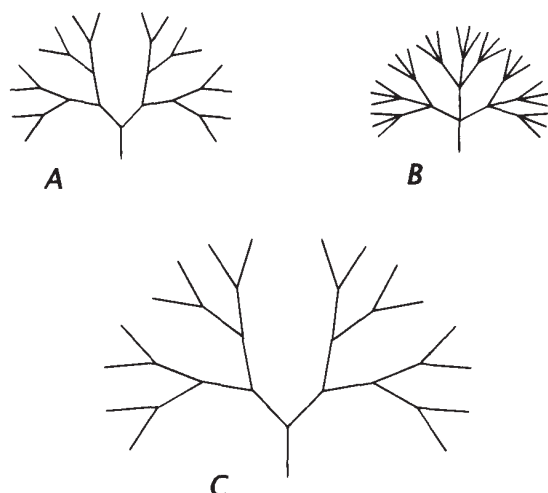
Fig. 4. Schematic drawing of different molecular designs of glycogen to show some physical properties of the molecule. A more realistic structure of glycogen is shown in Figure 1; in this picture some structural features are shown simplified in order to emphasize the influence of the branching degree and the chain length on the density in the most external tiers. The concept of tier has a *functional*—and no geometric—meaning as each stage in the metabolic synthesis or degradation of the molecule; that is, the most external tier is formed by those chains directly available to phosphorylase, although not all of them have necessarily their ends in the surface of the molecule (see Fig. 1); the action of phosphorylase on the most external chains realizing glucose, leaves room allowing the enzyme to work on more internal chains of the same tier. (A) Design with branching degree r = 2. (B) Design with branching degree r = 3. (C) Design with branching degree r = 2, but with chains longer than (A). Each of these designs has different physical properties as the density of the molecule in each tier and the amount of glucose available for phosphorylase. For example, comparing A with B it can be seen for higher values of r that the density of the tiers increases faster with the number of tiers.

Fig. 5. Optimization function if glycogen is synthesized under excess of the branching enzyme activity. The optimization function [f_{opt} = $(G_T \cdot G_{PT} \cdot C_A)/V_S$] is discontinuous and has an absolute maximum for $r = 2$, $g_c = 13$, $t_{max} = 12$. This result takes into account all physical constraints of the molecule building (density of every tier and maximal possible density in the last tier).

by overexpression of the branching enzyme. These facts are interesting because they demonstrate that an optimized structure of glycogen can be guaranteed under different conditions of synthase activity. The building of glycogen with *Case A* would force a more difficult regulation mechanism, as the two enzymes should be controlled coordinately to avoid changes in the activity ratio.

3. The distance between branching points is surprisingly constant, and so, branches appear on the residues 5 and 10 (see Goldsmith et al. 1982); that is, as near as possible from each other according the physical constraints. This means that the branching enzyme is in excess because any increase of its activity cannot produce a more dense (more branched) molecule.

Now, the next question is how is the chain length determined in the growth of the molecule? In Figure 2B the ends of each chain are marked by black points, but there is no special mechanism described to specify it, so the growth pattern should itself account for it. One possibility is that it can be determined by the ratio of activity between the two enzymes. The density of the glycogen
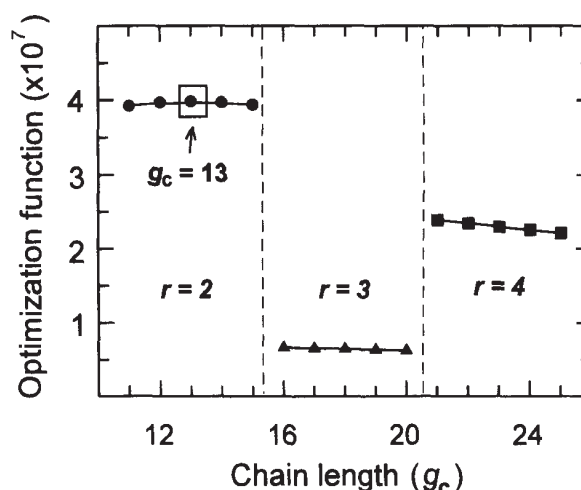
synthesized could define the limit of a growing chain, as the near branches could obstruct the work of synthase. There are two empirical facts that support this mechanism: (a) the results reported by Smith (1968) studying glycogen synthesis *in vitro* with phosphorylase (standing in for glycogen synthase, working in the reverse direction) at limited activity and variable amounts of the branching enzyme, demonstrated that the chain length was shorter under increasing amounts of the branching enzyme (having allowed its concentration range to change to a much lower value than that found in cells, so it could act like an independent variable); and (b) the opposite effect has also been reported *in vivo:* in glycogen storage disease type IV (Andersen disease) the activity of the branching enzyme is very low, with no change in synthase activity, and it produces a glycogen molecule with long external chains (Mercier and Whelan 1970). This fact is also well explained by the theory presented here: under a low activity of the branching enzyme, the pattern of glycogen synthesis is converted from *Case B* into *Case A,* with longer chains allowed.

We can thus conclude that the ratio of activities between branching enzyme and synthase account for the control of the glycogen chain length. This problem is different according to the size of the molecule, as in external tiers density is higher than in the inner ones (see Fig. 4). Consequently, if the mechanism to build the glycogen molecule were the same during the whole process, then the ratio between the two enzymes would be constant at any stage of glycogen growth, and then the inner chains should be longer than the external ones. This, however, disagrees with the experimental results which show that the inner chains have the same length as the external ones, or even shorter (Manners 1957; Man-

ners and Wright 1962; Bullivant et al. 1983). On the other hand, since the optimization function maximizes the glycogen density (G_T/V_S), this decline of density in the most inner tiers would cause a defective optimization of the molecular design.

The solution of this problem can be found in results reported by Whelan's group (Lomako et al. 1991, 1993; Alonso et al. 1995). According to these results, glycogen synthesis occurs in two stages: the first one is from glycogenin to an intermediate molecule of glycogen with 8 tiers and a molecular weight of $\approx$400,000 MW called *proglycogen;* and the second one from proglycogen to the full molecule with 12 tiers and a molecular weight of $\approx 10^7$ MW also called *macroglycogen.* Furthermore, Alonso et al. (1995) have suggested that the regular metabolism of glycogen only involves the conversion between proglycogen and macroglycogen. Taking into account the molecular weights of proglycogen and macroglycogen, we derive with our mathematical model that this turnover only involves the last four tiers. This difference of metabolism between external and internal tiers of the molecule could be a reasonable mechanism, since the stored fuel in the inner tiers is really little (about 6% of the full glycogen).

Optimization of the Glycogen Molecule: Is Half a Glycogen Necessary?

The mechanism of glycogen synthesis discussed in the former section can also explain the way its structure was optimized. In effect, that mechanism explains that the structure of glycogen can be designed (and thus optimized) just by means of controlling the activity of the enzymes involved in its synthesis. The function of optimization presented above in Eq. (5) depends on three independent variables: the branching degree r, the chain length g_c, and the number of tiers t. Let us now analyze them: t is an independent variable, and as a molecule of glycogen can have more or fewer tiers independently from the value of other parameters (and it really occurs during glycogen metabolism), but the maximal possible number of tiers t_{max} depends on other features of the molecule. It has been previously shown (Goldsmith et al. 1982; Madsen and Cori 1958; Meléndez-Hevia et al. 1993) that glycogen has t = 12 tiers because this is the limit that density imposes with r = 2 and g_c = 13, (cellular parameters which are precisely those that maximize f_{opt}). However, this limit could be different for other values of g_c or r. A glycogen with longer chains could have a higher branching degree or a bigger size with more tiers. Here, we shall assume the same physical constraints that operate in the cell: the permitted density, and the minimal number of glucose residues between branches, as the enzyme needs a certain amount of room to work. Considering the density of the 12th tier in cellular glycogen (463 glucose residues per nm^3) as the

maximal permitted, a glycogen molecule with a longer chain length (g_c = 17) and with the same branching degree r = 2 can have one more tier. The value of its optimization function (see Fig. 3) is higher when compared with the solution found by Meléndez-Hevia et al. (1993), that is,

$$f_{opt}(r = 2, g_c = 17, t = 13)$$
$$> f_{opt}(r = 2, g_c = 13, t = 12).$$

The same happens when r = 3: a glycogen with r = 3 and g_c = 16 can have t_{max} = 7 tiers, but one with r = 3 and g_c = 22 can have t_{max} = 8 tiers and then,

$$f_{opt}(r = 3, g_c = 22, t = 8)$$
$$> f_{opt}(r = 3, g_c = 16, t = 7).$$

Thus, the values of the optimization function in such glycogens are higher than that in the cellular one; longer chains produce less dense molecules, and one more tier is permitted, having more glucose available to phosphorylase. This fact is what causes the discontinuities in the plot of Figure 3, which shows these optimization functions.

One can, then, wonder, why has natural selection not chosen a longer chain which would have allowed one more tier? Darwin's theory states that any complex structure evolves toward its optimum in many small steps, but all of them needing to have a positive selective value. One of the most classical objections to Darwin's theory was how to explain the production through natural selection (by means of small changes) of highly complicated organs or structures, such as a wing or an eye, formed by a combination of several different pieces, when the usefulness of each piece separately had no obvious meaning. Discussing this problem Darwin (1859) stated "if it could be demonstrated that any complex organ existed which could not possibly have been formed by numerous successive slight modifications, my theory would absolutely break down." *The eye problem* presented there by Darwin is a classical representative example of this subject. There is now a general consensus about the eye evolution through small steps to give the highly evolved organ of present octopus or vertebrates [see, e.g., Dawkins (1986, 1994); Ridley (1993)]. Darwin's statement can be expressed in a mathematical way saying that natural selection is an algorithm that can only produce continuous functions. Now, making a paraphrase of the famous sentence of Mivart, remembering his classical objection to that evolutionary mechanism, we could wonder "what is the use of half a glycogen?" The key is the necessary role of the intermediate stages in the structure building process, and it can be applied to the glycogen puzzle. The meaning and physiological significance of the intermediate steps in synthesizing and depleting the glycogen molecule are clear because half a glycogen is really very useful, as it plays a physiological

role. When the molecules of glycogen are full, with 12 tiers, the directly available fuel is 34.6% of the total stored glucose, that is, about 19,000 glucose residues per glycogen molecule. This available glucose is also contained in the last tier of each full glycogen molecule, and it accounts for about 20 seconds of high-performance anaerobic exercise (fast movement) in the white muscle Type II fibers (cf. Newsholme and Leech 1983 (chapter 9); Peter et al. 1972; Thayer et al. 1993; Lupiáñez et al. 1996). Once that tier has been depleted, the glycogen available in the next tier (the 11th one) is also 34.6% of the stored glucose (about 9,500 glucose residues per glycogen molecule) which can support 10 additional seconds of quick movement, and this series continues about four times until the level of proglycogen which has 8 tiers. Thus the structure of *all tiers* must be optimized, because each one has a physiological meaning. It would have been nonsense for natural selection to operate on a fraction of the problem, leaving out other parts which must have the same fitness; if such a glycogen had been selected, then it would have been equivalent to selecting half an eye.

Let us now look at the differences between the structures corresponding to $(r = 2, g_c = 17, t = 13)$, and $(r = 2, g_c = 13, t = 12)$. Figure 3 and Eq. (6) show that the value of $g_c = 13$ gives a maximum for f_{opt} independently from the number of tiers t, that is, it is a maximum for every tier, this property being independent from the branching degree (any value of r). However, the solution with $g_c = 17$ is never a maximum at any tier, although $f_{opt}(g_c = 17, t = 13, r = 2) > f_{opt}(g_c = 13, t = 12, r = 2)$. Then a glycogen with $g_c = 17$ is a bad solution in each of its 12 first tiers, and its advantage appears only at the 13th tier. The path for obtaining a potentially better glycogen necessarily passes through a bad glycogen. Moreover, such a glycogen would be better just at its most external tier (at $t = 13$), and it would become a worse glycogen once its most external tier had been depleted. Natural selection cannot explain the production of a glycogen potentially better just in its 13th tier when its building would have required the selection of nonoptimized intermediate steps. We can conclude from these results that a glycogen with $g_c = 17$ has not had the opportunity to be selected. This shows the importance of the path to reach a solution. An end can only be selected if intermediate steps have been able to be selected. The glycogens with parameters $(r = 3, g_c = 16, t = 7)$ and $(r = 3, g_c = 22, t = 8)$, which are also shown in Figure 3, can be discounted on the basis of the same reasoning. Thus, our results show a clear case of achieving an optimized molecule through the optimization of each intermediate step.

Discussion

In the glycogen molecule, the branching degree $r = 2$ is the maximal possible value, as it is imposed by other parameters (the chain length and the physical constraints); indeed, since the optimal chain length is $g_c = 13$, the branching degree must be $r = 2$ because $r = 3$ is not possible with chain lengths of $g_c < 16$. Now, regarding the left panel in Figure 5, we can see that only values of g_c between 11 and 15 are allowed under these constraints. Experimental data of chain lengths obtained by several different groups for 60 years, using very different techniques, find precisely these values (Table 1); natural selection has thus solved a nice problem of optimization, and this fact also suggests some new reflections. Since different tissues and organisms have arrived at the same solution, this demonstrates empirically the generality of the optimization function, that is, all glycogens, no matter if from either different tissues in animals or bacteria or yeast have the same structure, showing that they are the result of an optimization process drived by the same fitness goal. At first sight it may not seem obvious that glycogen from white muscle—typical for quick response in regard to glucose mobilization—has the same fitness goal as that from slow-response organs as red muscle or liver. However, the reality is that in all these cases a quick response is also necessary. This statement is supported by the ubiquitous fast-trigger mechanism of the cascade that regulates phosphorylase activity. This conclusion can be also extended to "slow" and "quick" animals. On the other hand, plants lack the cascade mechanism, and their form of glucose storage (starch) is very different from glycogen, for example, average chain length of $g_c = 22$–25 (Manners and Wright 1962).

As has been discussed in the previous section, the function described in Figure 3 must be discounted, and thus we have concluded that the actual optimization function of glycogen is the one described in Figure 5. It can be seen that the solution of it involves that g_c must be in the interval of 11–15 including these limits. In other words, $g_c \in [11, 15]$ is really a unique solution. Of course, it does not mean that a problem of optimization has not existed, because the general problem can have many different solutions, as Figures 3 and 5 do show (in fact, glycogen storage disease type IV is a good empirical example of the fact that glycogen with a different structure can exist, and also shows that such a glycogen is bad!), but when the system is set in the mode of excess of the branching enzyme activity, then the finding of the optimal solution is easy. It is not difficult to think that some regulatory constraint—as mentioned above—could have taken part in the establishment of this condition; then, it would have accelerated the process of searching for the optimal solution. The high ratio of activities between branching enzyme and synthase (about 200-fold) seems to indicate that the increase of such a ratio has been well favored in the evolution of metabolic design.

We would like to emphasize the physical aspect of this problem and its solution. Our analysis demonstrates

that a single chemical machinery cannot build a fully optimized glycogen molecule because the physical constraints that control chain length are different in the inner and in the external zones; thus, if there were just a single mechanism to build the glycogen molecule along its full extension, then the chains in the inner tiers could be longer than the most external chains, which is contrary to experimental results (Manners 1957; Manners and Wright 1962; Bullivant et al. 1983). Therefore, two different tools are necessary to build the full molecule of glycogen, one for each zone. This is clearly a physical problem, since the two enzymes work with the same chemical mechanism. On the other hand, the intervention of the two enzymatic tools could generate a problem of metabolic regulation if both enzymes were involved in the regular glycogen turnover. In fact, only the external tiers are involved, and this practically does not affect the capacity of glucose store, since the fuel in the 8 inner tiers amounts to just about 6% of the full capacity, but it simplifies the metabolism. There is not much information about details of these two distinct glycogen synthases, although Farkas et al. (1991) have reported two isoforms of the enzyme in yeast, coded by distinct genes that are differentially controlled. Further results in this field will surely be the key to a final elucidation of this problem.

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