# TEMPERATURE-DEPENDENCE OF THE ACTION OF Q-ENZYME AND THE NATURE OF THE SUBSTRATE FOR Q-ENZYME

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## 1. Introduction

The 1,6-branch linkages that interlink the unit chains of amylopectin are formed by the action of Q-enzyme (EC 2.4.1.18) [1]. The most extensively studied variety of this enzyme is from potato, also the subject of this report. The enzyme acts by transglycosylation and we have demonstrated that this event can occur by inter-chain transfer of a chain fragment from a donor chain to an acceptor chain, a 1,4-bond being broken in the donor chain and a 1,6-bond being formed between the transferred fragment and the acceptor chain [2]. Intra-chain transfer, in which the donated portion is added to the residue of the chain from which the fragment was severed, has not been excluded.

There must obviously be minima to the lengths of the donor and acceptor chains. One of us reported some 20 years ago that when phosphorylase was allowed to elongate short maltodextrin chains in presence of Q-enzyme, action of the latter was not seen until the chains had reached an average chain-length ($\overline{CL}$) of about 40 [3]. The experiment did not permit a decision to be made whether the minimum length so observed was that of the donor, or the acceptor chain, or both. This observation has remained a paradox. An extended chain of 40 1,4-linked $\alpha$-glucose units would be 170 Å in length. This is considerably longer than the maximum possible size of the combining site of Q-enzyme (mol. wt about 85 000) [4].

With the demonstration that Q-enzyme acts by inter-chain transfer (see above) we have come to realize the possible significance of the otherwise puzzling observation. Inter-chain transfer could occur by successive actions of Q-enzyme, in which it first removes the donating chain fragment, and then seeks an acceptor substrate. Alternatively, the substrate for the enzyme may be a complex of two chains, so aligned together that the scission of a 1,4-bond and formation of a 1,6-branch linkage occur as a single, concerted and uninterrupted event. The postulate of French [5] that amylose chains, long known to be capable of adopting a helical configuration, can exist in a double helix, allows one to picture the substrate for Q-enzyme as such a double helix, and to describe the action of the enzyme as one of randomly 'stitching' the two chains to each other through branch linkages. If it is postulated as the only likely possibility that the adhesion between two chains is through hydrogen bonds, the paradox of the minimum $\overline{CL}$ 40 substrate for Q-enzyme becomes explicable. It is not until the chains reach this length that a complex (double helix?) is formed with sufficient stability and half-life to submit to the 'stitching' action of Q-enzyme.

This report provides two tests of the above hypothesis. First, the half-life of a complex between chains of any given length should increase as the temperature of the solution of chains is decreased. Therefore, the minimum length of chain on which Q-enzyme will act to introduce branches should not be an absolute value, but should decrease with temperature.

Secondly, French [5] has demonstrated by model building that a branch linkage between two chains offers little distortion to double helix formation between those chains. It should follow that once two chains have become joined by a branch linkage, and can no longer part company, the likelihood of a further branching action, or multiple actions occurring between

the two chains, will be increased, as will be the rate of branch point formation. Therefore, it may be predicted that if Q-enzyme acts on amylose in the absence and in the presence of a branching enzyme, added in order to split the 1,6-bonds as fast as they formed, a difference will be seen in the rates and extents of branching action in the two digests. If the hypothesis is correct, the presence of debranching enzyme will serve to lower the rate of action of Q-enzyme, and the extent of branching that Q-enzyme can introduce will also be lower.

## 2. Materials and methods

Amylose of degree of polymerisation 260 was purchased from Nutritional Biochemicals, and α-D-glucose 1-phosphate from Sigma. Maltotetraose was prepared as by Taylor and Whelan [6]. Potato Q-enzyme was purified to protein homogeneity and assayed as by Borovsky et al. [4]. It was free from α-amylase (EC 3.2.1.1), D-enzyme (EC 2.4.1.25) and pullulanase (EC 3.2.1.41). Potato phosphorylase was prepared as by Kamogawa et al. [7], the method being modified to include collection of the enzyme as an ammonium sulfate fraction (0.33–0.55 saturation) following heat treatment, and the use of an aminobutyl-Sepharose column fractionation [8] in place of DEAE-cellulose. The enzyme activity was 250 nkat/mg of protein and it was free from α-amylase, pullulanase, Q-enzyme and D-enzyme. Pullulanase was prepared from *Aerobacter aerogenes* and assayed as by Mercier et al. [9].

Digests in which Q-enzyme was incubated in the presence of a chain-extending system (phosphorylase, glucose 1-phosphate and maltotetraose) contained the following final concentrations of components: sodium citrate buffer, pH 7.0 (50 mM), maltotetraose (0.45 mM), glucose 1-phosphate (40 mM), phosphorylase (25 nkat/ml) and Q-enzyme, when present, (1.1 nkat/ml). The temperature of incubation was 35 or 4°C. Iodine staining was conducted by making a 21-fold or greater dilution of the digest into a solution of iodine–potassium iodide in which the final concentrations were 0.004% iodine and 0.04% iodide. Absorbance was measured at 680 nm. Inorganic phosphate was measured as by Huijing et al. [10].

Digests in which Q-enzyme acted on amylose ($\overline{DP}$ 260) in the presence and absence of pullulanase contained the following final concentrations of components: sodium citrate buffer, pH 7.0 (100 mM), amylose (1.6 mg/ml), Q-enzyme (0.11 nkat/ml) and pullulanase, when present, (8.3 nkat/ml). The temperature of incubation was 30°C. Iodine stain absorbance at 680 nm was measured at intervals as above. Measurement of the extent of branching was made at intervals by removing 0.5 ml digest portions, heating at 100°C for 3 min., and incubating with added pullulanase (final concentration 23 nkat/ml) at 30°C for 19 hr. Reducing power was measured by a modification of the Nelson method [11]. The reducing power, compared with appropriate controls, was used to calculate the number of branch linkages formed by Q-enzyme and subsequently hydrolyzed by pullulanase. In the digest where Q-enzyme and pullulanase acted together on amylose, there was no increase in reducing power during the subsequent incubation with pullulanase.

## 3. Results and discussion

### 3.1. *Minimum substrate chain length for Q-enzyme*

The original experiment of Peat et al. [3], to determine the minimum chain length at which Q-enzyme will initiate branching, was repeated at 35 and at 4°C. The experiment consists in comparing the properties of polysaccharides synthesized by phosphorylase from maltotetraose and glucose 1-phosphate in the absence and presence of Q-enzyme. The principal property under test is the intensity of iodine stain of the polymer. The chains grow by multi-chain action [12] and the $\overline{CL}$ at any point can be calculated from the amount of orthophosphate released from the Cori ester [3]. A plot of iodine stain absorbance versus $\overline{CL}$ can be constructed (fig.1). When Q-enzyme is present and branching begins to occur, the iodine stain absorbance at any particular value of $\overline{CL}$ is lower than that recorded in the absence of Q-enzyme because branching lowers the extinction coefficient. The onset of branching is therefore to be seen by the value of $\overline{CL}$ at which the curve for phosphorylase plus Q-enzyme begins to fall away from that for phosphorylase alone. It is seen that at 35°C this occurs at $\overline{CL}$ 30 (fig.1). In the original version of this experiment [3] the diversion was recorded at $\overline{CL}$ 40. There is no significant discrepancy here in view of the fact that in the present experiments

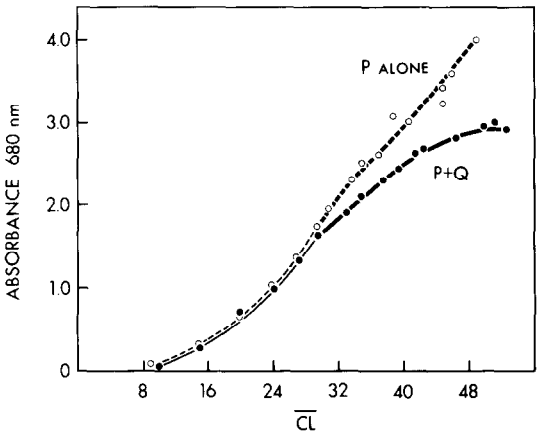


Fig.1. Action of potato Q-enzyme on growing amylose chains at 35°C. Potato phosphorylase was incubated with and without potato Q-enzyme as described in Materials and methods. Samples were withdrawn at intervals for measurement of iodine stain absorbance (680 nm) and inorganic phosphate. The latter value was used to calculate $\overline{CL}$ of the synthetic amylose [3,12]. The iodine-stain absorbance is calculated to that corresponding to a 1:20 mixture of digest with iodine-potassium iodide. The plot is of the iodine stain absorbance versus $\overline{CL}$ of the synthetic amylose, and shows the results for phosphorylase (P) alone (○) and phosphorylase plus Q-enzyme (●).

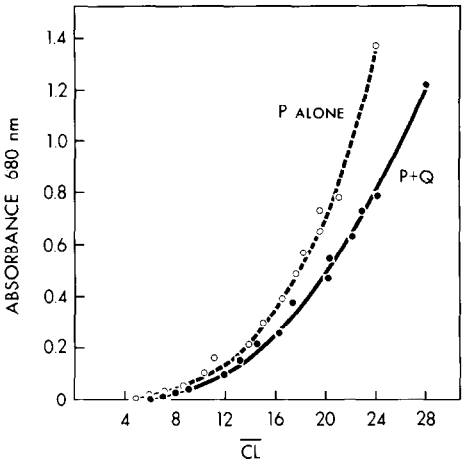


Fig.2. Action of potato Q-enzyme on growing amylose chains at 4°C. See fig.1 for details and explanation of symbols.

the relative amount of Q-enzyme to phosphorylase was 8 times higher than originally employed [3]. The minimum value of $\overline{CL}$ noted will necessarily fluctuate with the ratio of Q-enzyme to phosphorylase, and the lower value seen here is consistent with the relatively higher level of Q-enzyme employed.

When the experiment was repeated at 4°C, the predicted result was observed, namely that branching set in at a much lower value of $\overline{CL}$ than at 35°C (fig.2).

Two controls were necessary to validate this conclusion. First it had to be established that the pattern of phosphorylase action at 35 and 4°C, in respect of the relation between iodine-stain absorbance and $\overline{CL}$ is the same. This was verified. Secondly, if on lowering the temperature to 4°C the activity of Q-enzyme relative to phosphorylase were to increase, then, as noted above, the apparent minimum substrate chain length would decrease. When a comparison of the ratio Q-enzyme: phosphorylase activity was made, the value at 4°C was 45% of that at 35°C. Hence the drop in the minimum substrate chain length noted on lowering the temperature from 35 to 4°C, is probably even greater than is seen in the comparison made in figs.1 and 2.

Finally, in confirmation of the validity of the iodine stain test as a indication of the onset of branching, a second test for branched material was made, as also by Bailey et al. [3]. This was to heat inactivate the enzymes from time to time, add β-amylase and note whether there was a residual iodine stain, indicative of a β-limit dextrin, and hence of branched polysaccharide. When only phosphorylase was present, no limit dextrin was, of course, to be seen. When Q-enzyme was present, limit dextrin formation was noted at 4°C well in advance of the rate of accumulation at 35°C.

### 3.2. *Action of Q-enzyme on amylose in the presence and absence of pullulanase*

The rationale behind this experiment was explained in the introduction. The predicted effects of including pullulanase in a digest of Q-enzyme and amylose were observed. The rate of reaction, measured by iodine staining, was significantly lower in presence of pullulanase (fig.3). Iodine stain is, however, but a qualitative guide to reaction rate. A direct and quantitative measurement was made by determining the increase in reducing power consequent on hydrolysing the branch linkages with pullulanase. That is, as a result of successive actions of Q-enzyme and pullulanase on the amylose, or as a result of the simultaneous presence

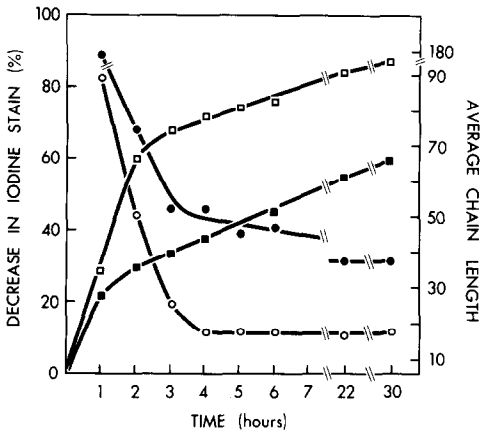


Fig.3. Action of potato Q-enzyme on amylose $\overline{DP}$ 260 in the presence and absence of pullulanase. Experimental details are given in Materials and methods. Iodine stain absorbance was measured at 680 nm and plotted as the percentage decrease in absorbance for Q-enzyme alone (□) and Q-enzyme plus pullulanase (■). At corresponding intervals the enzyme(s) in each digest were inactivated by heating and pullulanase was then added. The reducing power so developed was used to calculate the $\overline{CL}$ of the 1,4-α-glucan chains set free by pullulanase, and their $\overline{CL}$ is plotted in the figure for the incubation of amylose with Q-enzyme alone (○), and for amylose incubation with a mixture of Q-enzyme and pullulanase (●).

of both enzymes, the amylose was reduced to linear chains of ever shorter length, and the results have been plotted in this fashion (fig.3). What is seen is that the rate of branching is lowered in the presence of pullulanase, in agreement with the prediction that the introduction of branch linkages facilitates synthesis of further such linkages. Secondly, that Q-enzyme acting alone converts the amylose into a polysaccharide of $\overline{CL}$ about 20, as expected for the amylopectin end product. When pullulanase was present along with Q-enzyme, the corresponding value of $\overline{CL}$ was considerably higher, nearer to 40. Not only does the higher value confirm the predicted effect of pullulanase, but significantly, it is around the minimum substrate chain length found for Q-enzyme when the reaction system was one of chain lengthening (fig.1). That is, in a chain lengthening system Q-enzyme begins to act at around $\overline{CL}$ 40. In a chain shortening system, action ceases when the $\overline{CL}$ has dropped to 40.

## 4. Conclusion

The foregoing experiments gave results entirely consistent with predictions made on the basis that the substrate for Q-enzyme action consists of two chains that have become associated, probably by hydrogen bonding, and possibly forming a double helix, as pictured by French [5]. The experiments, and the conclusions, also emphasize the necessity for consideration to be given to secondary, tertiary and quarternary aspects of amylose and amylopectin structure. These higher orders of structure, and especially the interaction between polymer chains, have implications for the action of branching enzyme and perhaps other starch and glycogen metabolizing enzymes also. The experiments seem to demonstrate the reality of inter-chain association, whether as a double helix or in some other fashion. It seems reasonable to postulate that some enzymes such as Q-enzyme require associated chains as a substrate. The long known preference of muscle phosphorylase for a branched primer rather than a linear primer [13], potato phosphorylase does not show such a preference, may be an example where chain association is required. Other enzymes may act only on single chains. Any mechanism that regulated the relative amounts of single and associated chains could be a form of metabolic control.

The chain association concept of the substrate for Q-enzyme also has important implications for the structure of amylopectin, as will be discussed in a later communication.

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