# AN INSOLUBLE COMPLEX FORMED BY THE INTERACTION OF MUSCLE PHOSPHORYLASE WITH GLYCOGEN[1]

Zvi Selinger and Michael Schramm

Department of Biological Chemistry, The Hebrew University, Jerusalem, Israel

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Introduction: It has recently been shown that when glycogen was added to pancreatic $\alpha$-amylase in the cold, the enzyme formed an insoluble complex with the glycogen dextrins (Levitzki and Schramm, 1963). The interaction was essentially identical to that of an antigen-antibody precipitin reaction in that excess of either glycogen or enzyme* inhibited precipitation. It was therefore tentatively concluded that the enzyme and the substrate have each more than one combining site so that a macrocomplex containing many molecules of both enzyme and substrate is formed and precipitates. It seemed likely that such an interaction would also occur between muscle phosphorylase and glycogen since Madsen and Cori mentioned appearance of turbidity and agglutinated particles when muscle phosphorylase a was mixed with phytoglycogen (Madsen and Cori, 1958, see also, De La Haba, 1962).

The present communication demonstrates that muscle phosphorylase a as well as b interact with soluble glycogen to form an insoluble enzyme-substrate complex. As in the $\alpha$-amylase system, formation of the insoluble complex is prevented by excess glycogen.

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* Unpublished results.

Experimental and results: When muscle phosphorylase a was incubated* with glycogen, part of the enzyme bound to glycogen was precipitated. The amount of insoluble complex formed was dependent on the phosphorylase/glycogen ratio in the system, the absolute concentration of the reactants, the temperature and incubation time. Enzyme was not precipitated when dextran, levan or yeast mannan replaced glycogen. Table 1 shows that up to 62% of the enzyme could be precipitated with glycogen during incubation overnight at 0° in the standard test system (see under Table 1). It is further demonstrated that excess glycogen inhibits formation of the insoluble complex. With glycogen in larger excess than shown in Table 1, precipitation was completely prevented. The phosphorylase/glycogen ratio in the reaction mixture affected the composition of the insoluble complex but only to a limited extent. Glycogen from oyster and phytoglycogen from sweet corn showed the same quantitative results as demonstrated in Table 1 for rabbit liver glycogen. Since the non-reducing end groups of this highly branched polysaccharide represent about 6% of its weight (Stetten and Stetten, 1958), it is calculated that one enzyme molecule (M.W. 500,000) is bound per 25 end groups when the phosphorylase/glycogen weight ratio in the precipitate is 7.5 (see Table 1).

The washed precipitated complex, containing 1.5 mg enzyme/mg glycogen, showed a solubility of 6 μg protein/ml at 0° and 70 μg protein/ml at 30°. With a different batch of crystalline phosphorylase a the solubility at both temperatures was three times higher. Dissolution at 0° was not increased at pH 5 (acetate buffer) or pH 9 (Tris buffer). At pH 4 the complex dissolved immediately with concomitant loss of enzymic activity. Addition

* The buffer was 20 mM sodium glycerophosphate pH 6.8 containing 1.5 mM EDTA, unless otherwise specified.

Table 1

Precipitation of the muscle phosphorylase a glycogen complex as a function of the enzyme/glycogen ratio

| Glycogen in reaction mixture | Glycogen precipitated | Phosphorylase precipitated | Phosphorylase/glycogen weight ratio | |
|---|---|---|---|---|
| | | | in reaction mixture | in precipitate |
| μg | μg | % of total | | |
| 36 | 8 | 13 | 13.0 | 7.5 |
| 72 | 33 | 36 | 6.5 | 5.0 |
| 180 | 107 | 56 | 2.5 | 2.4 |
| 360 | 180 | 62 | 1.3 | 1.6 |
| 720 | 175 | 47 | 0.7 | 1.2 |
| 1440 | 146 | 29 | 0.3 | 0.9 |

Rabbit muscle phosphorylase a (Cori et al., 1955) recrystallized 3 times in presence of EDTA was diluted to 0.1% in 0.02 M sodium glycerophosphate buffer pH 6.8 containing 1.5 mM EDTA. Heating to 30° ensured complete dissolution of enzyme. Insoluble material, if present, was removed by centrifugation. A solution of rabbit liver glycogen (Mann Research Laboratories) was purified by treatment with ion exchange resins as previously described (Loyter and Schramm, 1962). The 'standard test system' in a final volume of 1 ml contained, 460 μg phosphorylase a, various amounts of glycogen reagents, 20 μmoles sodium glycerophosphate buffer pH 6.8 and 1.5 μmoles EDTA. The mixture was incubated overnight at 0° to ensure maximal precipitation and was then centrifuged in the cold at 2300 x g for 5 min. The supernatant and the precipitate, washed once in 1 ml of the glycerophosphate EDTA buffer, were analyzed for protein (Lowry et al., 1951), enzymic activity (Cori et al., 1955) and total sugar (Dubois et al., 1956). Precipitated enzyme was fully active when diluted for assay.

of 5 μg crystalline pancreatic α-amylase to the suspended precipitate containing 300 μg phosphorylase a, caused complete dissolution of the complex at 0° within a few minutes. It is therefore evident that only high molecular weight glycogen can maintain the insoluble complex with phosphorylase while its degradation products formed by α-amylolysis, are ineffective. Although the maximal yield of insoluble complex was obtained after overnight incubation at 0°, considerable amounts of enzyme precipitated with glycogen already

after 1 h (Table 2). The rate of precipitation appears to be higher at 0° than at 30°. However, the composition of the complex was apparently not affected by the incubation time or temperature, being determined solely by the enzyme/glycogen ratio in the reaction mixture. When the weight ratio in the reaction mixture was 1.2-1.3, incubation overnight at 0° or

Table 2

Effect of AMP and temperature on precipitation of phosphorylase a during 1 hour incubation

| Exp. | Temperature of incubation | Mononucleotide added | Phosphorylase a precipitated | Phosphorylase/glycogen Weight ratio in precipitate |
|---|---|---|---|---|
| | | | μg | |
| I | 30° | None | 150 | 1.7 |
| | 30° | AMP, $10^{-5}$ M | < 20 | - |
| II | 0° | None | 220 | 1.6 |
| | 0° | AMP, $10^{-6}$ M | 60 | - |
| | 0° | AMP, $10^{-5}$ M | < 20 | - |
| | 0° | XMP‡, $10^{-5}$ M | 180-220 | 1.6 |

‡ The following 5' mononucleotides were tested: IMP, GMP, UMP, TMP, CMP.

In exp. I 1000 μg phosphorylase a and 800 μg shellfish glycogen reagent were incubated for 1 h and analyzed as described under Table 1. The data were obtained by analyses of the supernatant after removal of the insoluble complex by centrifugation at room temperature. Exp. II was performed under the same conditions but at 0°, with 500 μg phosphorylase a and 400 μg glycogen.

for 1 h at 0° or 30°, yielded precipitates which were all of about the same composition (see Tables 1 and 2). It is also demonstrated in Table 2 that AMP, which specifically accelerates the catalytic reaction, strongly in-

hibits formation of the insoluble complex while all other 5' mononucleotides are inert.

Phosphorylase b also interacted with glycogen to form an insoluble complex (Table 3). The yield of enzyme precipitated was smaller

Table 3

Precipitation of muscle phosphorylase b with glycogen as a function of $Mg^{++}$ concentration

| Compound added | mM | Phosphorylase precipitated % of total | Phosphorylase/glycogen Weight ratio in precipitate |
|---|---|---|---|
| EDTA, | 2.0 | 20 | 1.1 |
| $Mg^{++}$, | 0.1 | 35 | 1.1 |
| $Mg^{++}$, | 1.0 | 50 | 1.3 |
| $Mg^{++}$, | 10.0 | 80 | 1.5 |
| AMP, | 1.0 | 0 | - |

Crystalline phosphorylase b was freed of AMP by treatment with Norit (Fischer and Krebs, 1958). Enzyme was diluted in glycerophosphate buffer as described for phosphorylase a. The system, incubated overnight at 0° contained, 500 μg phosphorylase b, 400 μg shellfish glycogen, 20 mM sodium glycerophosphate buffer pH 6.8 and magnesium acetate or EDTA as shown in the Table. The final volume was 1 ml. Isolation of the precipitate and analyses were carried out as described under Table 1.

than with phosphorylase a but could be increased considerably by addition of $Mg^{++}$. AMP on which phosphorylase b is dependent for catalytic activity was not required for the formation of the insoluble complex. Moreover, 1 mM AMP completely prevented precipitation of the enzyme with glycogen. As with phosphorylase a, precipitation of phosphorylase b was inhibited by excess glycogen. When the reaction mixture described in Table 3 contained

500 μg enzyme and 2000 μg glycogen, in presence of 10 mM magnesium, no insoluble complex was formed.

Discussion: Formation of the insoluble phosphorylase-glycogen complex might be due to two different types of interaction. 1) Several phosphorylase molecules bind to a single glycogen molecule. 2) Phosphorylase and glycogen, both having several combining sites, form a framework in which many molecules of both reactants are linked to each other. Mechanism 2 is identical to that of the antigen antibody precipitin reaction and may lead to the formation of a complex having a particle weight many times higher than that possible by mechanism 1. It would therefore appear more likely that mechanism 2 operates in the precipitation of the phosphorylase-glycogen complex but a final conclusion cannot be drawn from the data presented.

Apart from the interest in this precipitation phenomenon as such, it also appears to be a useful tool to study binding of phosphorylase to glycogen independent of the overall catalytic reaction. It was shown that AMP strikingly inhibited formation of the insoluble complex with both phosphorylase a and b. A more detailed study with phosphorylase a demonstrated that AMP specifically inhibited formation of the insoluble complex at a concentration equimolar to that of the enzyme ($10^{-6}$ M). Thus it is evident that the effect of AMP manifests itself already at the stage of binding of the enzyme to glycogen and not only in the overall catalytic reaction. It might therefore be postulated that the nucleotide imposes on the protein a modification of its binding sites for glycogen.

Since the phosphorylase a glycogen complex is formed at relatively low concentrations of the reactants and is quite insoluble at 30°, the existence of such a complex in the living cell should be considered. One

might indeed visualize that phosphorylase and UDPG glycogen synthetase (Leloir and Goldemberg, 1960), interact with glycogen to constitute a metabolically active subcellular particle.

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