Reversible Oxidation of *Azotobacter vinelandii* Polynucleotide Phosphorylase

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(Received 13 January 1969)

Olmstead & Lowe (1959) showed that trypsin-treated *Micrococcus lysodeikticus* polynucleotide phosphorylase was inhibited by iodoacetate or, less reproducibly, PCMB*. These authors suggested that free thiol groups in the protein molecule were required in the polymerization reaction. Their results have recently been confirmed and extended (Klee & Singer, 1968). Further, the preferential loss of the CDP-polymerization activity during carefully limited trypsin degradation (Fitt & Fitt, 1967), or the general inactivation and increase in primer requirement caused by more extensive proteolysis (Fitt & Fitt, 1967; Klee, 1967; Klee & Singer, 1967; Fitt, Fitt & Wille, 1968), can be reversed by β -mercaptoethanol (Klee & Singer, 1968; Fitt *et al.* 1968; Klee, 1968), and thiol reagents have some effect on the undigested aged enzyme (Klee & Singer, 1968). However, no similar effects have yet been described with other polynucleotide phosphorylases, and Grunberg-Manago, Ortiz & Ochoa (1956) found that low concentrations of PCMB had little effect on the activity of the partially purified *Azotobacter vinelandii* enzyme. We now report that the highly purified *A. vinelandii* enzyme is inactivated by oxidation or treatment with thiol-binding reagents, and that the effect is reversible to a great extent. Some irreversible inactivation also occurs.

Highly purified *A. vinelandii* polynucleotide phosphorylase in the reduced form was prepared in good yield by an improved method (A. T. Gajda & P. S. Fitt, unpublished work). It migrated as a single sharp band of protein and activity during polyacrylamide-gel electrophoresis by the method of Fitt *et al.* (1968), and was at least 95% pure. The enzyme was assayed by the standard procedure, which measures incorporation of [14 C]nucleoside diphosphates into an acid-insoluble form (Fitt & Fitt, 1967; Grunberg-Manago *et al.* 1956). It was stored at 2–4° in the presence of 10 mM- β -mercaptoethanol and had no primer requirement for ADP incorporation, although CDP incorporation was stimulated 60% by ApA (1 mg./ml.). This preferential stimulation of polyC synthesis by added

primer is similar to that found in *M. lysodeikticus* polynucleotide phosphorylase after limited proteolysis (Fitt & Fitt, 1967). At all levels of purity, the fully reduced enzyme is slightly inhibited by β -mercaptoethanol, although the latter is required for stability during storage. A similar inhibition of impure preparations of *Escherichia coli* polynucleotide phosphorylase by thiols has been observed (Boguyavlenskaya, Guberniev & Shorosheva, 1967).

The slow inactivation of the enzyme by air was studied under controlled conditions (Fig. 1). The ADP-incorporation activity was fully retained for at least 1 day in buffer after removal of β -mercaptoethanol. However, during the next month, the activity decreased slowly, and a small β -mercaptoethanol requirement developed. At the same time there was a small but definite fall in the free thiol content of the enzyme as measured by the method of Boyer (1954). This suggests that the enzyme contains free thiol groups essential for activity.

The reduced enzyme could be oxidized in a rapid uncontrolled manner by adsorption on a DEAE-Sephadex column through which air was then passed for 5 hr. After elution the enzyme had lost 66% of its ADP-incorporation and 82% of its CDP-incorporation activities measured in the absence of primer. All the lost activity was restored by β -mercaptoethanol (100 mM, in the assay) but none by ApA. In fact, the primer requirement for CDP incorporation was the same as for the reduced enzyme, and ADP incorporation by both the reduced and oxidized enzyme was inhibited by ApA. Clearly, the inactivation of the fresh enzyme by oxidation was unrelated to the development of a primer requirement.

The reduced enzyme was sensitive to oxidizing agents such as *p*-benzoquinone. The reaction mixture contained 1 mM-tris-HCl buffer, pH 7.4, 1 mM- β -mercaptoethanol and 2 mM-*p*-benzoquinone and was kept at 2–4°. Samples were withdrawn at various times and assayed for ADP incorporation with and without 100 mM- β -mercaptoethanol. In these conditions *p*-benzoquinone virtually eliminates ADP-incorporation activity within 15 sec. However, up to 60% of the original activity was restored by the addition of β -mercaptoethanol to

* Abbreviations: ApA, adenylyl-(3'-5')-adenosine; PCMB, *p*-chloromercuribenzoate; polyA, polyadenylic acid; polyC, polycytidylic acid.

Fig. 1. Slow aerial oxidation of polynucleotide phosphorylase. The enzyme, in 10 mM-tris-HCl buffer, pH 7.4, containing 10 mM- β -mercaptoethanol, was dialysed at 2–4° against several changes of 10 mM-tris-HCl buffer, pH 7.4. The dialysis bags had been boiled in 1 M-NaOH and washed thoroughly. At the indicated times samples were withdrawn and assayed for ADP incorporation with and without 100 mM- β -mercaptoethanol. Thiol concentration was measured by the method of Boyer (1954). The incorporation assay procedure was as follows. The reaction mixture (final volume 0.1 ml.) contained: tris-HCl buffer, pH 9.0, 15 μ moles; $MgCl_2$, 1 μ mole; EDTA, 0.04 μ mole; [^{14}C]ADP, 4 μ moles (about 4000 c.p.m./ μ mole); enzyme. Incubation was at 37° for 15 min. The reaction was stopped by addition of 0.1 ml. of 7% (w/v) $HClO_4$ and the mixture was kept at 0° for 10 min. The precipitate was collected on a Whatman GF/C filter, washed four times with 1% (w/v) $HClO_4$, once with 50% (v/v) ethanol, dried and counted in a Nuclear-Chicago low-background thin-window counter. The residual activity of the treated enzyme is expressed as a percentage of the activity of the untreated enzyme measured under the same conditions. Δ , Activity without β -mercaptoethanol; $\bullet$, activity with β -mercaptoethanol; $\blacktriangle$, thiol concentration.

the assay medium. A similar result was obtained with potassium ferricyanide: the loss of activity was slower and less extensive than with *p*-benzoquinone and was completely reversed by β -mercaptoethanol.

The effect of PCMB on the reduced enzyme was then studied in the conditions described above. A slow incomplete inactivation occurred during 1 hr. and was reversible by β -mercaptoethanol. This was not a non-specific inhibition of the enzyme by PCMB: when the enzyme was treated with 2 mM-PCMB for 2 hr. and then dialysed against 500 vol. of buffer containing no β -mercaptoethanol all its

activity was lost; but in the presence of β -mercaptoethanol 50% of the original activity was recovered. Similar results were obtained with GSSG.

It is important to note that some of these changes are not completely reversible. Further, some slow changes not reversible by β -mercaptoethanol occur even in its presence. After the fresh reduced enzyme had been stored for 2 months with 10 mM- β -mercaptoethanol at 2–4°, it had lost 30% of its ADP-incorporation and 70% of its CDP-incorporation activities, measured in the absence of primer. These losses were completely reversed by ApA even though the enzyme was not stimulated by added β -mercaptoethanol, i.e. the enzyme had acquired a primer requirement for polyA synthesis and an increased primer requirement for polyC synthesis. It would seem that the 'irreversible' changes represent not a loss in synthetic ability but rather a loss in chain-initiating ability. This is analogous to the effect of extensive proteolysis on the activity of *M. lysodeikticus* polynucleotide phosphorylase.

Our results show that fresh *A. vinelandii* polynucleotide phosphorylase undergoes an inactivation by oxidation or thiol-binding reagents that is wholly or partly reversible by β -mercaptoethanol and is independent of changes in primer requirement. During storage in β -mercaptoethanol inactivation also occurs that is reversible by primer but not by β -mercaptoethanol. It is therefore possible that the development of a primer requirement is due to unspecified structural changes during aging, and not to the reversible oxidation of essential thiol groups, as has been suggested by Klee & Singer (1968) on the basis of studies with trypsin-treated aged *M. lysodeikticus* polynucleotide phosphorylase.

This work is supported by the Medical Research Council of Canada.

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