EFFECTS OF PHOTO-OXIDATION ON THE CATALYTIC AND REGULATORY PROPERTIES OF CITRATE SYNTHASE FROM *ACINETOBACTER LWOFFI*

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1. Introduction

The citrate synthases (EC 4.1.3.7) of Gram-negative bacteria have been shown to be sensitive to regulation by NADH [1]. As in studies of other regulatory enzymes, useful information may be gained from the selective modification of the catalytic or regulatory enzymic functions and, indeed, desensitization to regulatory effectors is the favoured operational criterion of an allosteric mechanism.

We have investigated the effects of photo-oxidation on the behaviour of citrate synthase from *Acinetobacter lwoffi*, a strictly aerobic Gram-negative bacterium, and have found that photo-oxidation results in desensitization of the enzyme to NADH inhibition. Under other conditions the enzymic activity may be destroyed by photo-oxidation and this has been investigated with both Methylene Blue and Rose Bengal as the photo-sensitive dyes. The cationic and anionic nature, respectively, of these two dyes gives rise to a hitherto unreported complementary pattern of pH dependence of photo-inactivation and our results suggest that specific interactions between dye and enzyme may be crucial for the photo-oxidation process.

2. Experimental

Citrate synthase was purified from nutrient-grown *A. lwoffi* by a procedure very similar to that previously described for *Escherichia coli* citrate synthase [2]. The purified enzyme had a specific activity of 80 μmoles of CoA.SH formed per min per mg of protein.

Unless otherwise stated, assays of enzymic activity were performed spectrophotometrically at 412 nm [3] in 0.1 M Tris–HCl, pH 8.0, with 0.2 mM oxaloacetate, 0.15 mM acetyl-CoA and 0.1 mM 5,5′-dithiobis-(2-nitrobenzoate).

Photo-oxidation was carried out with a 150 Watt spot-light positioned 20 cm from the reaction mixture contained in a conical glass centrifuge tube immersed in water maintained at 20°C. Buffer solution (50 mM phosphate at the appropriate pH) and enzyme (plus oxaloacetate where used) were introduced into the tube in a volume of 0.9 ml. The light was switched on and the photo-oxidation initiated by the addition of 0.1 ml of the photo-sensitive dye to give a final dye concentration of 3 μM. At intervals, samples were withdrawn and, to prevent further reaction, immediately diluted ten times with 0.1 M Tris–HCl buffer, pH 8.0, in small glass tubes covered with aluminium foil. The concentration of protein in photo-oxidation experiments was 0.1 mg/ml. Suitable control experiments were carried out in the absence, respectively, of dye, light or oxygen. In this way it was demonstrated that the various effects observed were the results of photo-oxidation of the enzyme. At the lower pH values examined, control rates of enzyme inactivation in the absence of dye were subtracted from the observed photo-inactivation rates.

3. Results and discussion

The effect of photo-oxidation on the catalytic activity was first investigated. Fig. 1 shows the time-

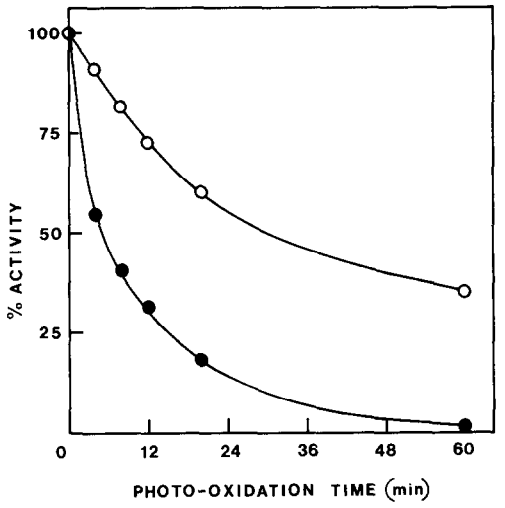

Fig. 1. Photo-inactivation of citrate synthase with Methylene Blue. Photo-oxidation was carried out as described in the text in 50 mM phosphate buffer, pH 7.0 (•), enzyme alone; (○), with 1 mM oxaloacetate.

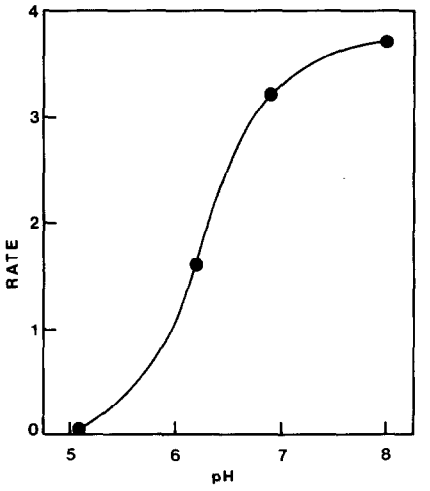

Fig. 2. pH Dependence of the rate of photo-inactivation with Methylene Blue. The relative rates, in arbitrary units, were obtained from the slopes of semi-log plots of the decay of enzymic activity.

course of photo-inactivation with Methylene Blue in 50 mM phosphate buffer, pH 7.0. The presence of substrate, 1 mM oxaloacetate, during the photo-oxidation resulted in considerable protection. Thus, for example, when the enzyme had been photo-oxidized for 20 min in the absence of oxaloacetate, less than 20% of the original activity remained whereas, in the presence of 1 mM oxaloacetate, 60% of the activity was retained (fig. 1).

The pH dependence of photo-oxidation with Methylene Blue is shown in fig. 2. The shape of this curve implicates a group with a pK_a in the region of 6.0–6.5 in the photo-inactivation and is therefore a tentative indication that histidine may be involved in the activity of the enzyme, though amino-acid analysis and other specific modification will be required to support this. With *E. coli* citrate synthase, photo-oxidation experiments also implicated histidine in catalytic activity and the results of amino-acid analysis and inactivation with diethyl pyrocarbonate supported this conclusion [4].

Rose Bengal was introduced as a photo-sensitive dye in the hope that its anionic structure would result in greater specificity for active sites that bind anionic substrates than that shown by the cationic Methylene Blue [5]. This hope was fulfilled in the case of yeast enolase [5] in which Rose Bengal was found to be far more efficient, and more specific for histidine residues, than Methylene Blue. Rose Bengal has subsequently been used in photo-oxidation studies on a large number of enzymes and proteins, e.g. [6–12].

We therefore examined the photo-inactivation of citrate synthase with Rose Bengal. Initial experiments were done at pH 8.0 and unexpectedly showed an inactivation rate very similar to that obtained with Methylene Blue. However, when photo-oxidation was done at lower pH, enhanced rates of inactivation were observed and fig. 3 shows the pH dependence of photo-inactivation with Rose Bengal. The shape of the curve suggests that a group with a pK_a in the region of 5.5–6.0 may be involved in the photo-inactivation and this might again be a histidine residue [13,14]. However, whereas the Methylene Blue experiments suggest that photo-oxidation occurs with the unprotonated residue, the results with Rose Bengal suggest that it is the protonated form which is more susceptible to photo-oxidation.

The photo-oxidation behaviour observed here with Rose Bengal is very unusual and, to our knowledge, only one other case of such behaviour has been reported [15]. It is generally believed that photo-oxi-

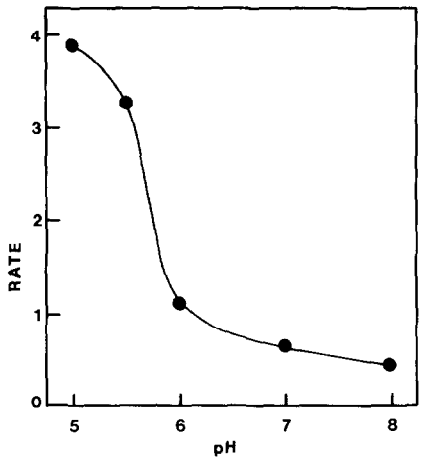

Fig. 3. pH Dependence of the rate of photo-inactivation with Rose Bengal. The relative rates, in arbitrary units, were obtained from the slopes of semi-log plots of the decay of enzymic activity.

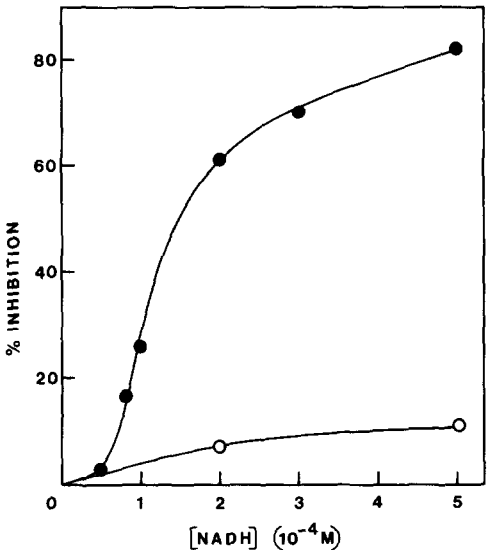

Fig. 4. Inhibition of native and desensitized citrate synthase by NADH. Assays were performed as described under Experimental but in buffer of composition 20 mM Tris adjusted to pH 8.0 with the disodium salt of EDTA. (•), native enzyme; (o), enzyme photo-oxidized in 50 mM phosphate, pH 7.0, 1 mM oxaloacetate, for 20 min with Methylene Blue.

dation of histidine occurs only when the imidazole group is unprotonated [5,16]. Moreover, with the exception of flavins, photo-sensitive dyes show increased rates of photo-oxidation with increasing pH, regardless of the charge on the dye [17,18]. Nevertheless, the interaction between the anionic Rose Bengal and histidine residues would be expected to be greatest when the latter are in the cationic protonated condition. The unusual pH dependence which we have observed is consistent with such an interaction and it may be that strong binding of the dye to the enzyme leads to a redistribution of charges which allows histidine to be photo-oxidized at low pH. The complementary pH dependences of photo-inactivation by the cationic Methylene Blue and anionic Rose Bengal observed in the present study constitute a novel dye-specific phenomenon. Further studies may show that this type of behaviour occurs with some other enzymes and may reveal that greater specificity of residue destruction is achieved at the lower pH values.

The marked protection by oxaloacetate against photo-oxidative destruction of enzymic activity has enabled us to study the selective photo-oxidation of the regulatory sensitivity to NADH. Photo-oxidation in the presence of 1 mM oxaloacetate and either Methylene Blue or Rose Bengal results in active enzyme almost completely desensitized to NADH inhibition (fig. 4). This selective desensitization clearly demonstrates the allosteric nature of the NADH regulation.

Examination of the dependence of enzymic activity on the concentrations of both acetyl-CoA and oxaloacetate indicated essentially identical behaviour for the native and desensitized enzyme. We have previously shown that those citrate synthases which are inhibited by NADH are considerably larger molecules than those which are insensitive to NADH [19]. It was therefore conceivable that desensitization had been accompanied by molecular dissociation. However, gel filtration experiments with the same marker proteins used previously [19] indicated no breakdown into smaller units.

In view of the different pH dependences of photo-inactivation with Methylene Blue and Rose Bengal reported above we examined photo-oxidative desensitization by these two dyes at various pH values. In contrast with the effects on enzymic activity, photo-oxidation with either dye in the presence of

1 mM oxaloacetate caused desensitization to NADH at rates which increased with increasing pH.

Phosphofructokinase from sheep heart [20] and phosphoenolpyruvate carboxylase from *E. coli* [21] have been desensitized to their regulatory effectors by photo-oxidation but, otherwise, the technique has been little used to desensitize regulatory enzymes though it is likely to offer a useful addition to the other methods employed. Indeed, in the present case of *A. lwoffi* citrate synthase, photo-oxidative desensitization is particularly valuable as urea, thiol-blocking reagents or heat – the frequently used desensitizing treatments – are ineffective. It has been shown that some dehydrogenases can bind dyes in competition with the nucleotide [8,22] and the possibility has been suggested [23] that dye-binding to nucleotide sites may be of general occurrence and photo-oxidation may thus be a useful tool in the examination of nucleotide-sensitive enzymes. The results presented here provide another example in support of this possibility.

Acknowledgement

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