ALTERATIONS OF ENZYMES BY RIBOFLAVIN AND BY BROMOPHENOL BLUE DURING PREPARATIVE DISC-ELECTROPHORESIS

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1. Introduction

Despite its extensive analytical application disc-electrophoresis on polyacrylamide gels is used only to a minor degree for preparative separations, probably because only rather limited quantities of protein can be separated and numerous shortcomings lead to relative low recoveries. However, preparative disc-electrophoresis may be well suited for the final stage of a purification procedure, provided that adequate recoveries can be attained. In the course of a systematic examination of preparative disc-electrophoresis on polyacrylamide gels as a tool for removing very low concentrations of contaminants from purified proteins, we observed the formation of artefacts, which considerably reduced the yield of the enzymes investigated. With D-galactose dehydrogenase from *Pseudomonas saccharophila*, an enzyme which is highly sensitive to oxidants, the characteristic oligomeric artefact-forms of the enzyme [1,2] were formed during preparative disc-electrophoresis. In this paper it is shown that riboflavin sensitized reactions and, under unfavourable conditions, Bromophenol-Blue-dependent inactivations may well account for the adverse effects noted.

2. Materials and methods

2.1. *Materials*

Riboflavin and Bromophenol Blue were obtained from Merck (Darmstadt, Germany). Acrylamide was recrystallized from chloroform at 50°C, *N,N'*-methylenebisacrylamide from acetone at 50°C [3]. All other substances were purchased from the usual commercial sources in the highest grade or purity available.

2.2. *Enzyme*

D-Galactose dehydrogenase was prepared from *Ps. saccharophila* and shown to be homogeneous in polyacrylamide gel electrophoresis and in sodium dodecyl sulfate electrophoresis [4].

2.3. *Analytical disc-electrophoresis*

Analytical disc-electrophoresis was performed according to Davis [5]. The enzyme samples were applied with or without a small concentration of Bromophenol Blue as a tracking dye. Controls without the addition of Bromophenol Blue were carried out for the experiments investigating the influence of riboflavin. Protein was stained with Coomassie Blue R 250 [6].

2.4. *Preparative disc-electrophoresis*

Preparative disc-electrophoresis was performed as vertical slab gel electrophoresis with end elution using Ultraphor (Colora Messtechnik GmbH, Lorch/Württ., Germany) electrophoresis equipment with a gel system according to Davis [5]. A 7.5 mm wide separation chamber was used. The separation gel had a height of 50 mm, the spacer gel of 40 mm. All solutions were used as described [7].

The activated protein [2] was equilibrated against spacer gel buffer with the aid of a Sephadex G 25

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column, and the solution (total volume about 12 ml; 3 mg protein per ml) was made 20% in glycerol and faintly blue with Bromophenol Blue. The solution was layered on the spacer gel with the aid of a peristaltic pump. During electrophoretic concentration the current was kept constant at 30 mA (150 V), for separation at 60 mA (400 V). Elution was performed at a flow rate of 42 ml/hr and fractions of 1.4 ml were collected. All procedures were performed at 6–8°C.

2.5. *Dye-dependent reactions with enzyme*

D-Galactose dehydrogenase in Tris–H_3PO_4 buffer (60 mM, pH 6.9), corresponding to the spacer gel buffer, was exposed to a light source (photopolymerizing light, Canalco, Bethesda, USA) in the presence of riboflavin or of Bromophenol Blue and in contact with air. For experiments to determine time dependency of enzyme inactivation the riboflavin concentration was 15 μM, the Bromophenol Blue concentration was 30 μM or 200 μM, respectively. Dark controls were performed.

2.6. *Determination of sulfhydryl groups*

The number of total sulfhydryl groups was determined with 5,5′-dithio-bis(2-nitrobenzoic acid) [8] in 0.1 M Tris–HCl buffer pH 8.0, containing 1 mM EDTA and 0.3% sodium dodecyl sulfate. The formation of 2-nitro-5-thiobenzoate was followed spectrophotometrically, measuring the absorbance at 412 nm and using the molar extinction coefficient of 13 600 M^{-1} cm^{-1} [8,9].

3. Results

3.1. *Preparative disc-electrophoresis*

The most prominent feature in preparative disc-electrophoresis of D-galactose dehydrogenase was the low yield of about 60% of enzyme activity. In order to trace this missing activity the separation gel was stained for activity and subsequently for protein, after the peak of D-galactose dehydrogenase activity had been eluted. A great number of characteristic bands (fig. 1), which all showed enzymatic activity,

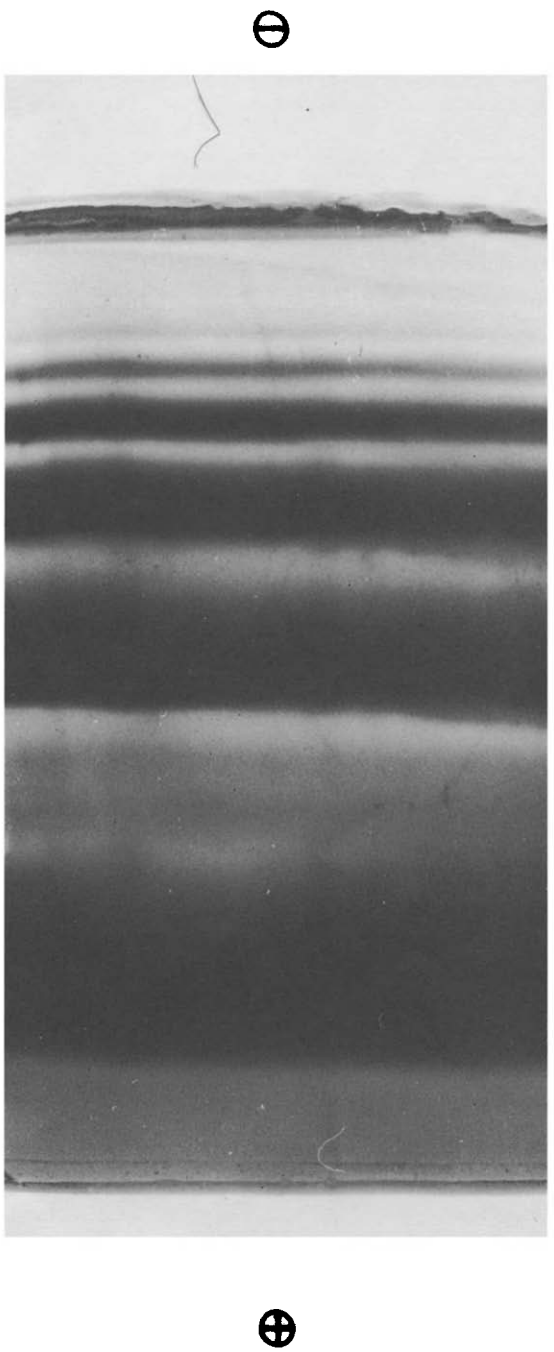

Fig. 1. Protein stained separation gel after preparative disc-electrophoresis. D-Galactose dehydrogenase with the molecular weight of 102 000 has been eluted. The visible bands correspond to oligomeric artefact forms of the enzyme.

Table 1
Riboflavin-dependent inactivation of D-galactose dehydrogenase

Riboflavin concentration	Specific activity after illumination	Specific activity after reactivation according to [2]
0	202 U/mg	202 U/mg
1.5 μM	202 U/mg	202 U/mg
5.0 μM	165 U/mg	190 U/mg
13.0 μM	123 U/mg	145 U/mg
48.0 μM	30 U/mg	69 U/mg

were found. Because the enzyme exhibited only a single band in slab electrophoresis [4] before the preparative run, a reasonable assumption is that the artefact-forms are generated during preparative disc-electrophoresis, presumably by oxidation and formation of disulfide bridges.

3.2. *Riboflavin-dependent alterations of the enzyme*

Flavin-sensitized photooxidations of amino acids are well known and many enzymes are photoinactivated in the presence of riboflavin, FMN or FAD [10–13]. In order to show qualitatively that the alterations of D-galactose dehydrogenase may be the result of a riboflavin-sensitized reaction, the enzyme was illuminated for 30 min at different riboflavin concentrations from 1.5 μM to 48 μM. The data in table 1 demonstrate clearly the influence of the riboflavin concentration on the inactivation of the enzyme. In the absence of riboflavin no loss of activity was observed.

Native D-galactose dehydrogenase (15 μM) illuminated in the presence of riboflavin (15 μM) showed a decrease in specific activity as well as in the number of sulfhydryl groups determinable under denaturing conditions (table 2). The inactivation followed first-order kinetics (fig. 2), although some photobleaching of riboflavin did occur during the exposure. The decrease in the number of sulfhydryl groups was accompanied by the formation of additional protein bands, migrating more slowly in electrophoresis than the native enzyme (fig. 3). By means of a dark control it was shown (gel 4 in fig. 3) that other causes cannot account for the observed additional bands. Neither an inactivation nor a loss of sulfhydryl groups was

Table 2
Time dependency of riboflavin-sensitized decrease of specific activity and of sulfhydryl groups

Time	Specific activity after illumination	Sulfhydryl groups/ mole enzyme
0	202 U/mg	11.3
30 min	145 U/mg	9.0
52 min	129 U/mg	–
71 min	111 U/mg	8.6
102 min	77 U/mg	–
110 min	–	7.8
158 min	48 U/mg	7.2
209 min	27 U/mg	–

observed in the presence of riboflavin when the solutions were kept in the dark.

Exposure to daylight in general causes the same results, and the lamp was only used for greater reproducibility.

3.3. *Reversibility of riboflavin-dependent alterations of the enzyme*

As expected, the treatment of photooxidized D-galactose dehydrogenase with 2-mercaptoethanol (0.1 M) or with 1,4-dithioerythritol (10 mM) at pH 8.6 resulted in the formation of one not very sharp protein band in electrophoresis, but only in a partial restoration of the original specific activity. It is evident

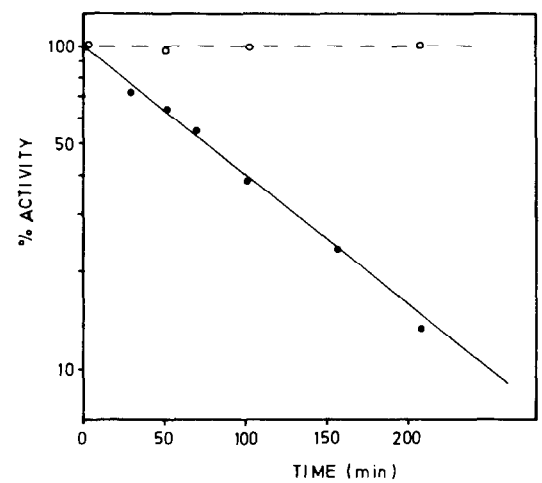

Fig. 2. The time course of riboflavin-sensitized (riboflavin 15 μM) photoinactivation of D-galactose dehydrogenase (15 μM).

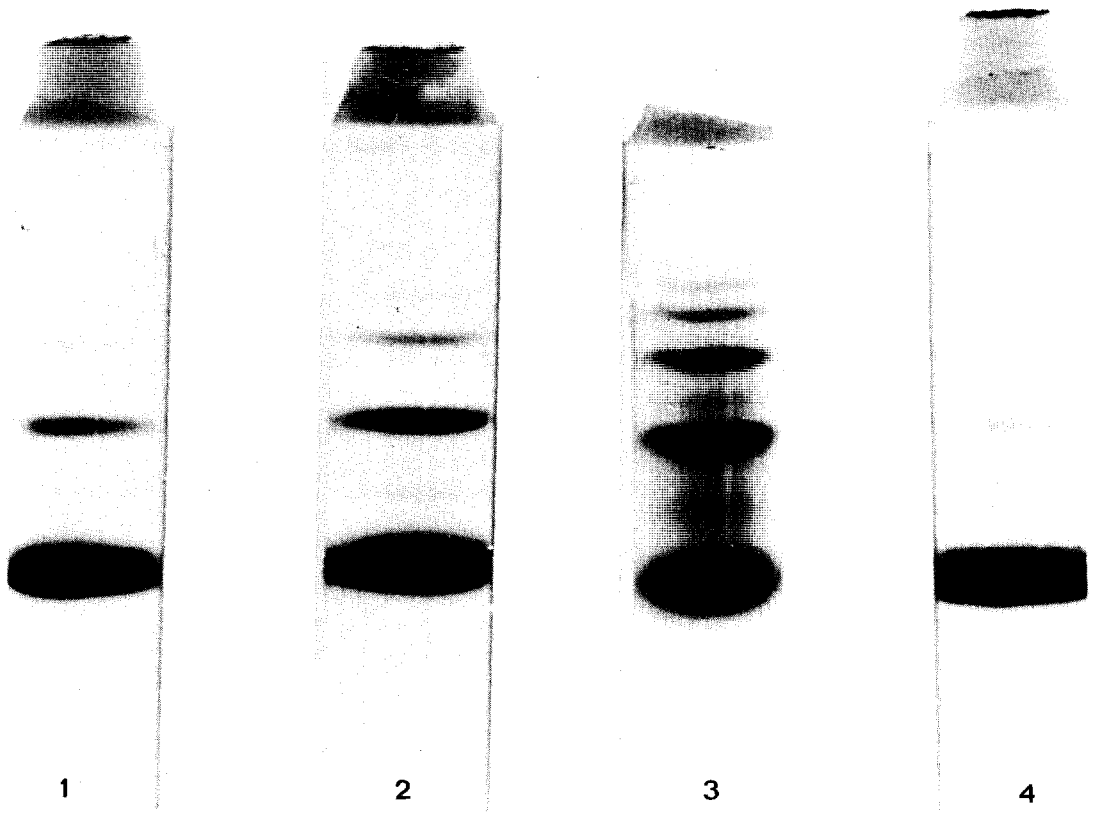

Fig. 3. Disc-electrophoretic separation of D-galactose dehydrogenase (20 μM) which had been exposed to light in the presence of riboflavin (15 μM) for different lengths of time: 1) 10 min; 2) 60 min; 3) 180 min; 4) dark control.

that together with the reversible oxidation of sulfhydryl groups other amino acid side chains had been irreversibly destroyed.

3.4. *Bromophenol Blue-dependent inactivation of enzyme*

Because Bromophenol Blue may give rise to the formation of artefacts [14] its influence on D-galactose dehydrogenase (20 μM) was examined at different dye concentrations (20–200 μM). The enzyme was readily inactivated by Bromophenol Blue at a concentration of 200 μM. This inactivation showed no dependence on illumination. The inactivation could not be reversed by the addition of 2-mercaptoethanol or 1,4-dithioerythritol, and gel electrophoresis revealed that the oligomeric artefact-forms of D-galactose dehydrogenase had not been formed. With a Bromophenol Blue concentration of 20 μM an inactivation could not be observed.

4. Discussion

Disc-electrophoresis on polyacrylamide gels is known to give rise to a number of artefacts. Thus the monomers, acrylamide and bisacrylamide, may interact with proteins [15], the catalyst $(NH_4)_2S_2O_8$ may produce artefacts [16, 17], Bromophenol Blue [14] and even buffer ions may cause artefacts [18, 19].

With D-galactose dehydrogenase from *Ps. saccharophila* we have obtained recoveries of only 60% in preparative disc-electrophoresis and observed the formation of artefact bands. It was determined that,

with the exception of Bromophenol Blue under certain conditions, the well-known causes could not account for the observed effects. Therefore we considered the possibility that the observed alterations might be attributable to riboflavin, which had been used for photopolymerization of the spacer gel.

With aldose-1-epimerase from *E. coli* we also became aware of a decrease in specific activity as the result of preparative disc-electrophoresis (K. Wallenfels, O. Blachnitzky and G. Kurz, unpublished). The enzyme from bovine kidney cortex is known to be rapidly photoinactivated in the presence of sensitizing dyes [20]. Riboflavin has now been shown to account for a first order photoinactivation of D-galactose dehydrogenase and for the formation of oligomeric artefact forms. The photoinactivation of the enzyme can be reversed only to a minor extent by treatment with 2-mercaptoethanol or 1,4-dithioerythritol. Hence, amino acids other than cysteine are probably involved.

The described alterations of enzyme may be of minor importance in analytical disc-electrophoresis, yet in preparative disc-electrophoresis recoveries of about 60% of the activity applied are an achievement worthy to note. With the equipment employed, either the conventional riboflavin photopolymerized spacer gel may be used and the electrophoretic run must be shielded from light, or a modification of the spacer gel may be used [21] in order to avoid the alterations noted.

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