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CROSSED AFFINITY IMMUNOELECTROPHORESIS OF THE *ESCHERICHIA COLI* PYRUVATE DEHYDROGENASE COMPLEX

JAAP VISSER* and HARRY C.M. KESTER

Department of Genetics, Agricultural University, Generaal Foulkesweg 53, 6703 BM Wageningen (The Netherlands)

SUMMARY

The crossed immunoelectrophoretic pattern obtained with the intact pyruvate dehydrogenase complex of *Escherichia coli* can be modified when this technique is combined with affinity gel electrophoresis using reactive dyes coupled to agarose as ligands. The patterns that arise have been interpreted with respect to localization of the three component enzymes. This was realized by using antibodies with different specificity, active enzyme staining and E_2—E_3 subcomplex behaviour. Dissociation of E_1 subunits occurs more easily than that of E_3 but remains incomplete in this system. The free reactive Procion Blue-MX dyes tested inactivate the complex even at neutral pH. The dyes react with all three components but E_3 (80%) and E_2 (15—20%) retain part of their catalytic activity. Modification leads to an enhanced dissociation of E_1.

INTRODUCTION

The pyruvate dehydrogenase complex is a large multi-enzyme complex that occurs in facultative and obligate aerobic bacteria. It catalyses the oxidative decarboxylation of pyruvate to acetyl coenzyme A (CoA) according to the following overall reaction: pyruvate + NAD^+ + CoA—SH → acetyl—S—CoA + CO_2 + NADH + H^+.

Among bacteria, the complexes of *Escherichia coli* [1, 2], *Azotobacter vinelandii* [3, 4] and of *Bacillus stearothermophilus* [5, 6] have been most thoroughly investigated. In the case of *E. coli*, the complex consists of multiple copies of three different enzymes, namely pyruvate dehydrogenase (E_1; EC 1.2.4.1) and lipoamide dehydrogenase (E_3; EC 1.6.4.3), which bind independently to dihydrolipoamide acetyltransferase (E_2; EC 2.3.1.12). Transacetylase E_2 forms the structural core of the complex.

The subunit stoichiometry of the *E. coli* complex has been a point of long debate and ratios of 2:2:1 [1, 2] as well as 2:1:1 have been reported [7, 8]. Recently, a stoichiometry of 1.5:1:0.5 has been proposed for the *A. vinelandii* complex [9], which may hold for *E. coli* as well.

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Immunoelectrophoretic methods have been used frequently to analyse multi-component systems [10, 11]. Thus far, however, no detailed immunological studies have been reported on the pyruvate dehydrogenase complexes, although they represent an interesting example of an interacting multi-component system. We have applied both crossed and crossed affinity immunoelectrophoresis, using reactive dyes as ligands, to analyse the complex of *E. coli* as reported previously [12].

The information obtained gives relevant information with respect to the integrity of the complex as well as to the effects of certain mutations.

EXPERIMENTAL

Organisms

The pyruvate dehydrogenase complex of *E. coli* mutant K1-1 LR8-13, kindly provided by Dr. U. Henning, was grown aerobically at 37°C in complete medium adjusted to pH 7.0 containing 10 g/l Bacto peptone (Difco), 5 g/l yeast extract (Difco), 5 g/l sodium chloride, 1 g/l glucose and 50 mg/l thymine. The deletion strain CΔ42 (aroP-aceE)Δ42, derived from K1-1 LR8-16, which lacks the E_1 gene was provided by Dr. J.R. Guest and was grown as described [13]. The bacterial cells were harvested by centrifugation and washed with saline before storage at −60°C.

Enzyme purification

Purification of the pyruvate dehydrogenase complex of *E. coli* followed the procedure outlined previously [14, 15]. The complex was dissociated under alkaline conditions for the selective isolation of E_1 [1, 16], whereas E_3 was also purified according to Reed and co-workers [16, 17]. The overall activity and the partial enzyme activities were measured as described previously [16, 18].

Immunology

Antibodies were prepared against the intact pyruvate dehydrogenase multienzyme complex and also against the pyruvate dehydrogenase (E_1) and lipoamide dehydrogenase (E_3) components. All antibodies were raised in male New Zealand White rabbits. The animals were injected twice subcutaneously using Freund's adjuvans complete with a ten-day interval. Ten to twelve days later this was followed by an intravenous booster. Blood was collected repeatedly over a period of several months. During this time span, an additional intravenous injection of antigen was given. The sera were freeze-dried and stored at −20°C.

Preparation of dye—agarose matrices

Reactive dyes were coupled to agarose (0.8%), which was dissolved by heating in Veronal buffer [pH 8.6, ionic strength (μ) = 0.05] by adding 100 mg of a reactive dye per g of agarose. After 5 min, the pH was raised to 10.5—11.0 by using a 1 *M* sodium carbonate solution. The coupling reaction was continued for 1 h at 55°C, then the pH was taken back to 8.6 and the agarose was poured into Petri dishes to give a thin gel layer (3—4 mm). The gels were

cut in pieces and extensively washed with Veronal buffer until no dye was released. They were finally stored at 4°C in Veronal buffer at pH 8.6. The gels could be dissolved easily by heating before gel-casting on glass plates.

Crossed immunoelectrophoresis and crossed affinity immunoelectrophoresis

From a 0.8% (w/v) dye—agarose solution in Veronal buffer (pH 8.6, μ = 0.05), 15 ml were cast on a glass plate (8.2 × 9.2 cm). After setting of the gel, 6-μl samples were applied to holes (0.25 mm) punched out of the gel. Electrophoresis was performed for 1—1.5 h at 5 V/cm at 15°C. After the transfer of gel slices to agarose-precoated glass plates, the gels for the second dimension were prepared by pouring an additional 10 ml of an antibody-containing agarose solution onto each plate. Electrophoresis was performed at 4 V/cm at 15°C for 3 h in a direction perpendicular to electrophoresis in the first dimension. After washing away the excess of protein, the precipitates were stained for 10 min in a solution containing 0.1% (w/v) Coomassie Brilliant Blue R250 in an ethanol—acetic acid—water mixture (45:10:45). Destaining occurred using the same solvent.

Active enzyme staining in situ

Residual lipoamide dehydrogenase activity present within the immunoprecipitate was detected in the following way. After electrophoresis, the gel was pressed and then rinsed with a 0.1 *M* potassium phosphate buffer (pH 7.0). The gel was then incubated at 25°C in a 0.2 *M* potassium phosphate buffer containing 1.2 m*M* nicotinamide-adenine dinucleotide, oxidized (NAD) and 0.6 *M* reduced lipoamide. After 10 min, the reactants were removed and the gel was immersed in buffer containing 0.07 m*M* 8-dimethylamino-2,3-benzophenoxazin (Meldola Blue) and 0.2 m*M* 3-(4,5-dimethylthiazolyl-2)-diphenyltetrazoliumbromide (MTT). Owing to the presence of reduced lipoamide, a background reaction occurred, so the pattern was only developed for 5 min. After rinsing with buffer, a second staining cycle could be applied if necessary. Finally, fixation was done after rinsing with buffer in a 5% (v/v) acetic acid solution. The advantage of this procedure compared to other electron acceptors is that Meldola Blue is neither light-sensitive nor autoxidable [19, 20].

Sodium dodecyl sulphate-polyacrylamide gel electrophoresis (SDS-PAGE) and Western blotting

PAGE was performed according to Laemmli [21] using 7.5% gels. Proteins were transferred electrophoretically to nitrocellulose (BA85; Schleicher and Schüll) without pretreatment of the polyacrylamide gels. A Bio-Rad transblot apparatus was used and the transfer was done at 40 V for 18 h at 4°C using a buffer containing 25 m*M* Tris and 192 m*M* glycine (pH 8.3) and 20% (v/v) methanol according to the manufacturer's instructions. After transfer, saturation of the nitrocellulose sheets with bovine serum albumin (BSA) and subsequent incubation with the appropriate antibodies were performed according to Zabel et al. [22]. Finally, the blots were extensively washed and then incubated for 2 h at room temperature with a sheep anti-rabbit immunoglobulin G peroxidase conjugate. After further washing, the blot was finally

stained at room temperature using (per 100 ml of a Tris–HCl buffer, pH 7.5) 30 μl of hydrogen peroxide (30%) and 40 mg of 4-chloro-1-naphthol dissolved in 1.5 ml of 96% ethanol. The colour usually developed in 10–30 min and the reaction was stopped by washing several times in distilled water that had been acidified by a drop of acetic acid.

RESULTS

Crossed immunoelectrophoresis of the E. coli pyruvate dehydrogenase complex

The antiserum prepared against the intact complex contained mainly antibodies directed against E_1 and E_3, but Western blotting also indicated the presence of E_2-specific antibodies. The anti-E_1 and anti-E_3 antibodies were also specific with respect to their corresponding antigens, as shown in Fig. 1. The different antibodies were tested in this experiment with crude extracts prepared from *E. coli* strain K1-1 LR8-13 and the E_1 deletion strain CΔ42. This experiment confirmed the absence of the E_1 protein in the latter strain. The crossed immunoelectrophoretic patterns of the purified *E. coli* multienzyme complex obtained with antibodies raised against the intact complex and against the individual components are shown in Fig. 2. The profile with the anti-complex antibodies consisted at least of two precipitin lines, with a diffuse zone in between to be seen most clearly at the skewed, tailing edge of the pattern. No complete equilibration of all reaction lines is obtained, owing to the fact that the individual components are still assembled within the complex. The pattern is characteristic for the state of assembly of the complex*.

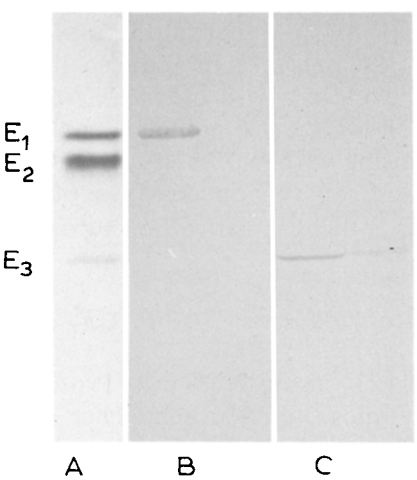

Fig. 1. Western blots of the wild type *E. coli* and a deletion mutant CΔ42 pyruvate dehydrogenase complex separated by SDS-PAGE. Crude bacterial extracts were used. From left to right, the following antibodies were used: anti-complex antibodies (A) only wild type, anti-E_1 (B) and anti-E_3 (C) antibodies.

*Storage of purified complex in a diluted form, which caused partial dissociation of the complex, did split up and sharpen this pattern.

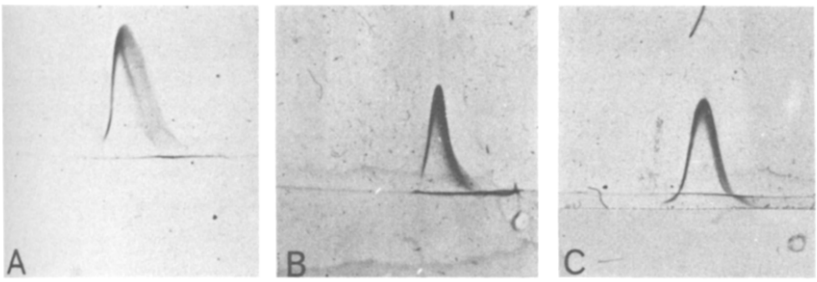

Fig. 2. Crossed immunoelectrophoresis of purified pyruvate dehydrogenase complex. A protein solution of 0.3 mg/ml was used, whereas the following antisera were used: anti-complex, 0.5% (A), anti-E_3, 0.25% (B) and anti-E_1, 0.5% (C).

With the component-specific antibodies against E_1 and E_3 using the intact complex, sharper reaction lines were obtained. The displacement of precipitin lines upon addition of a component specific antibody to the anti-complex antibodies could be used to identify the outer and inner borders of the precipitin zone as being mainly E_1 and E_3 reaction lines, respectively.

Crossed affinity immunoelectrophoresis of the E. coli complex

Analysis of the interaction of various dyes with individual components present in the multi-enzyme complex was initiated for two reasons. The first reason was related to whether one could influence the state of integrity of the complex by its interaction with dyes. If differential binding would occur, the changes resulting from this were then thought to allow identification of individual subunits in the precipitation pattern. As an analytical application of this, the routine screening of mutants to investigate their complex structure in crude extracts was considered.

The second reason was to screen these dyes to evaluate their potential as ligands in preparative affinity chromatography of subcomplexes or individual components.

When applying dye—agaroses as intermediary gel in the second dimension of a crossed immunoelectrophoretic experiment, we observed that the original precipitation pattern of Fig. 2A was split up into several well defined reaction lines. Some dyes were then further tested in the first dimension and turned out to affect the precipitation patterns in a similar way. Three characteristic examples are shown in Fig. 3, illustrating the effect of Procion Blue MX-3G, Procion Blue MX-R and Mikacion Brilliant Yellow 6GS, respectively. In the presence of these dyes, the two main precipitin lines became separated (cf. Fig. 2A). The tailing of the precipitin lines and the various peaks which arose in the inner precipitate reflected the interaction of individual components or subcomplexes with the various dyes. All further experiments were done to enable the interpretation of these patterns. The same interactions were studied again but this time analysed with component-specific antibodies. In the case of anti-E_1, in all cases fused reaction patterns with a double peak were obtained as shown for Procion Blue MX-R and Procion Blue MX-3G (Fig. 4A and B). This identified at least two different forms of E_1. Part of the E_1 present in this precipitate must be derived from E_1 chains which appeared in a com-

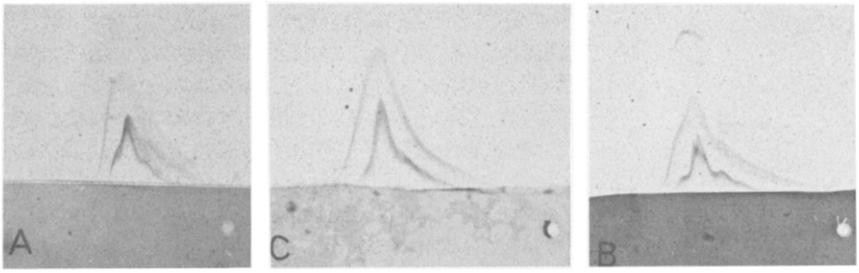

Fig. 3. Crossed affinity immunoelectrophoresis of the purified *E. coli* pyruvate dehydrogenase complex. A protein solution of 0.3 mg/ml was used. The following dyes were used in the first-dimension agarose gel: Procion Blue MX-3G (A), Procion Blue MX-R (B) and Mikacion Brilliant Yellow 6GS (C). Anti-complex antiserum (0.5%) was used in the second dimension.

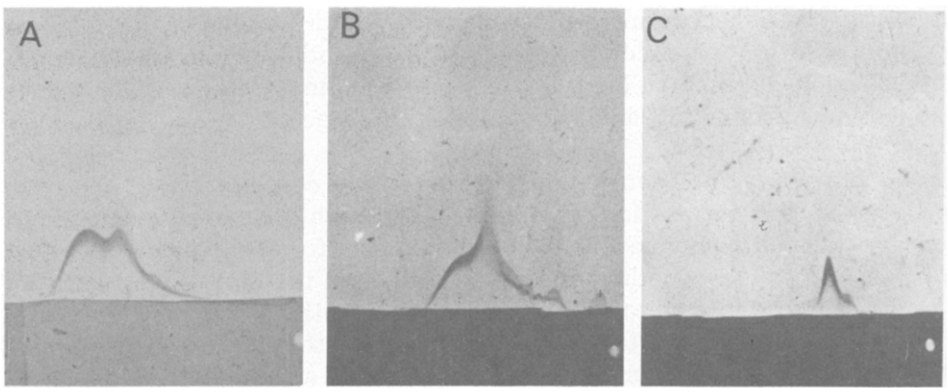

Fig. 4. Crossed affinity immunoelectrophoresis of the *E. coli* pyruvate dehydrogenase complex. A protein concentration of 0.3 mg/ml was used. The following dyes were applied in the first dimension: Procion Blue MX-3G (A) and Procion Blue MX-R (B and C). In the second dimension, 0.5% anti-E_1 antiserum (A and B) and 0.25% anti-E_3 antiserum (C) were used.

plex form in the inner precipitate when analysed with anti-complex antibodies (cf. Fig. 3). Complex formation still occurs and interferes with complete equilibration. The top of the newly obtained E_1 peaks corresponded in the case of all three dyes that were analysed with the migration position of the extreme right-hand side small peak in the inner profile (cf. Fig. 3).

With anti-E_3 the shape of the inner profile obtained with anti-complex antibodies returned to a large extent as illustrated in the case of Procion Blue MX-R (Fig. 4C). The leading edge of the inner precipitate, which only appears using anti-complex antibodies (Fig. 3B), most likely represents a reaction product of anti-E_2 and the core protein. The presence of E_2 in the leading edge would also explain why in the anti-E_3 precipitation reaction the precipitin line suddenly breaks off and does not return to the baseline (Fig. 4C). An excess of core protein certainly disturbs proper equilibration between E_3 and anti-E_3, thus preventing the formation of a continuous precipitation line. The presence of E_3 was also confirmed by active enzyme staining, which was applied in the crossed affinity immunoelectrophoretic pattern

with Procion Blue MX-R using anti-complex antibodies for analysis. The pattern obtained was identical in shape to that in Fig. 4C.

Analysis of the E_2—E_3 subcomplex

Analysis of subcomplex behaviour offers another possibility for further characterization of the reaction patterns. By using the E_2—E_3 subcomplex we could ascertain the position of E_2. This subcomplex was not prepared from the constituent components; we simply used an *E. coli* strain CΔ42 carrying a deletion of the E_1 gene. In Fig. 5A, the precipitation pattern shown is obtained with anti-complex antibodies. A leading reaction line was found that partially fused with a more slowly migrating predominant peak. Using anti-E_3 antibodies for analysis leaves only this second precipitate (Fig. 5B). This feature compares well with the previously mentioned observations using dye-mediated electrophoresis and justifies the suggestion made previously about the origin of the leading edge of the inner precipitate in Fig. 4C.

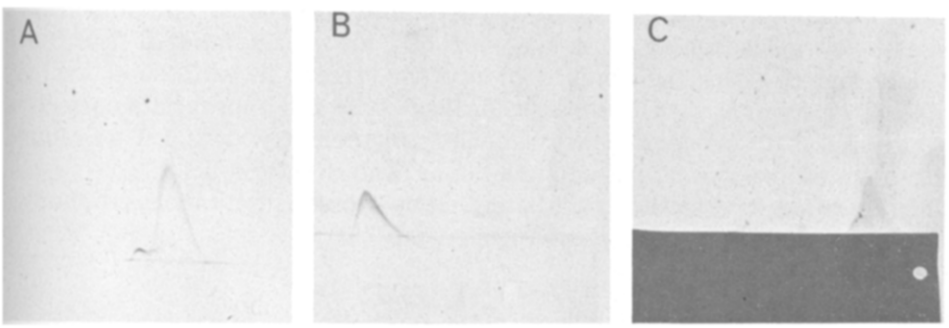

Fig. 5. Crossed immunoelectrophoresis of the *E. coli* pyruvate dehydrogenase complex, mutant CΔ42. A crude bacterial extract was used, whereas the following antisera were used: anti-complex, 0.5% (A and C), anti-E_3, 0.3% (B). In the case of C, Procion Olive MX-3G was used in the first dimension.

Interestingly enough, we were able to change the fused reaction lines (Fig. 5A) into separate reaction lines by using Procion Olive MX-3G as the affinity ligand in the first dimension (Fig. 5C).

Analysis of dye-modified E. coli complex

It has been noticed that reactive dichlorotriazine dyes, when exposed to some enzymes at slightly alkaline pH, behave as site-directed irreversible affinity labels [23]. We noticed that the *E. coli* pyruvate dehydrogenase complex was even inactivated at pH 7.0 by a number of reactive dyes, such as Procion Blue MX-3G and Procion Blue MX-R. These reactive dyes bound to all three components, as determined by optical analysis after SDS-PAGE. We were, of course, interested to see in which way dye modification of the protein would interfere with crossed affinity immunoelectrophoretic analysis of the complex. On the basis of kinetic measurements at various dye concentrations, a Procion Blue MX-R concentration was used that inactivated the enzyme complex completely within 1 h at 25°C (Fig. 6). The dye did not inactivate all components to the same extent; the partial residual enzyme

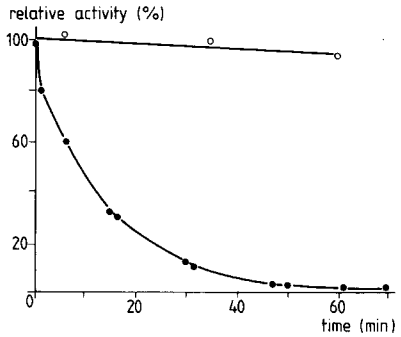

Fig. 6. Inactivation of the purified *E. coli* pyruvate dehydrogenase complex by Procion Blue MX-R. The enzyme (0.4 mg/ml) was inactivated by adding ca. a 400-fold excess of reactive dye at pH 7.0 and at 25°C. The relative decrease in activity was determined with respect to a control preparation kept at 25°C.

activities measured were 15—20% for E_2 and 80% for E_3, as compared to the unmodified complex. The amount of dye present in each component was estimated by gel scanning at a λ_{max} of 597 nm and a ratio of 7.5—8:2:1 was determined. Also, the amount of protein present in each band was calculated after staining with Coomassie Brilliant Blue and appropriate correction for dye—ligand absorbancy. Since a chain stoichiometry of 2:1:1 was found in this preparation, dye was bound in a molar ratio of 3.7—4:2:1.

This modified enzyme was then analysed by crossed immunoelectrophoresis using all three available antibodies.

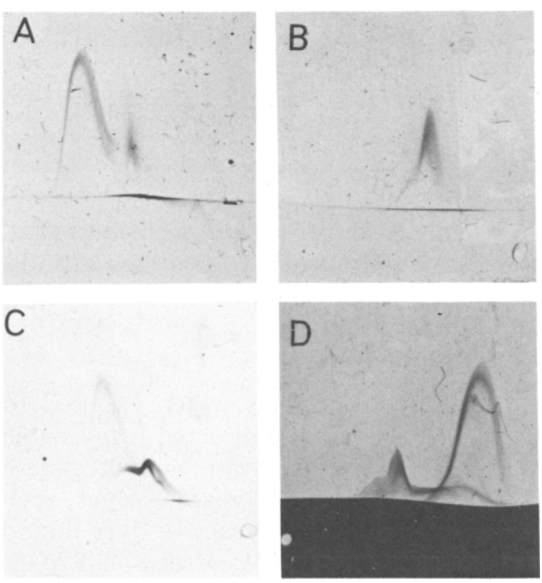

Fig. 7. Crossed immunoelectrophoresis of pyruvate dehydrogenase complex modified by Procion Blue MX-R. Inactive multi-enzyme complex (0.13 mg/ml) was used, whereas the following antisera were applied: anti-E_1, 0.5% (A), anti-E_3, 0.25% (B) and anti-complex, 0.5% (C). In D (opposite migration direction), the inactivated complex was used at a concentration of 0.27 mg/ml, whereas anti-complex antiserum (0.5%) was applied. In the first dimension, Procion Blue MX-R agarose was used.

With the anti-E_1 antibody, two reaction peaks were obtained that did not fuse and were abruptly cut off (Fig. 7A). A single, slightly asymmetric peak corresponding in position with the minor E_1 peak was observed when anti-E_3 antibodies were used for detection (Fig. 7B). With the anti-complex antibodies, a more complicated pattern was obtained, as a third peak arose at a position in-between the two peaks already observed (Fig. 7C). Both the leading E_1 peak and the new peak fuse with the third peak. The most reasonable explanation for these observations is that the leading peak is representing dissociated E_1, which at the tailing edge becomes associated with E_2. The core protein is the predominant component in the reaction peak underneath the E_1 reaction line, and this precipitate now fuses on its turn with the third peak. Since we know that E_1 and E_3 are present in this peak, this precipitate is most likely to contain all three components.

When Procion Blue MX-R agarose was applied in the first dimension, again using anti-complex antibodies to develop the crossed immunoelectrophoretic pattern, the E_1 precipitate became completely separated (Fig. 7D), whereas E_2 and E_3 still formed a fused reaction pattern.

DISCUSSION

When the purified pyruvate dehydrogenase complex of *E. coli* was submitted to a crossed immunoelectrophoretic analysis, both the skewed pattern and the diffuse precipitin zone at the tailing edge indicated slight differences in composition across the reaction boundary. This was found regardless of whether antibodies against one or more components were used. Interaction of the complex with reactive dyes, immobilized in the agarose gel, led, however, to resolution of several reaction lines in which individual components or subcomplexes were present. By destabilizing the subunit interactions within the complex, these dyes upon binding may cause dissociation of subunits or subcomplexes, which are then separated in an electric field. In the second dimension, this would favour the formation of separate reaction lines with the different kinds of antibodies available. Although the patterns obtained with the various dyes are different when looking at the profiles in detail, the main features are the same.

Therefore, we concentrated our studies on the analysis of the interaction with Procion Blue MX-R as an example. The result is schematically shown (Fig. 8) and the presence of the various components has been tentatively indicated. This picture is based on arguments derived from the experiments described. The arguments for the presence of E_1 in the outer precipitate are

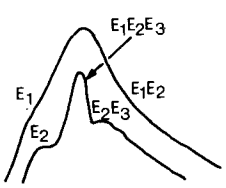

Fig. 8. Schematic representation of crossed immunoelectrophoresis of *E. coli* pyruvate dehydrogenase with interaction with Procion Blue MX-R, immobilized in the agarose gel.

based on the results obtained with component-specific antibodies (anti-E_1 and anti-E_3). The tailing edge of the outer precipitate appears to be a precipitin zone rather than a sharp precipitin line. We interpret this to be due to the presence of E_2, which, because of its interaction with E_1, perturbs final equilibration. Active enzyme staining has shown that the flavoprotein did not occur in the outer precipitate but that it is present everywhere in the inner precipitate except for the leading edge. The localization of E_3 is confirmed using anti-E_3 antibodies; in this pattern, a leading edge is lacking. Using two facts, we conclude that the leading edge is formed by E_2. This reaction boundary appears only when anti-complex antibodies are used that were proved to contain anti-E_2 in addition to antibodies against the other two subunits. Secondly, the same pattern was observed with the E_2—E_3 subcomplex present in a E_1 deletion mutant. Since the leading edge fuses with the central precipitation peak, E_2 is likely to be present also further down the pattern.

Although we have no direct evidence for the presence of E_1 in the two main peaks of the inner precipitate, there are indications for this. Firstly, there is an increase in peak height in the profile of the complex developed with anti-E_1. This occurs at a position corresponding to that of the small peak at the right-hand side (cf. Figs. 3 and 4). Secondly, crossed immunoelectrophoretic analysis of the multi-enzyme complex, which was covalently modified by the free reactive dye, indicates that E_1 is still present in two forms. The major part of E_1 dissociates and migrates fast. The position of the other form, however, coincides with that of E_3 and E_2, the presence of the latter being evident from a fused reaction line.

Thus, it looks as if even under these conditions part of the subunits still remain in a complexed form although we do not know of which size. The analysis and interpretation of the crossed affinity immunoelectrophoretic patterns obtained with the pyruvate dehydrogenase complex are useful to visualize rather quickly the effects of ligands, chemical modification, solvent composition, etc. on the integrity of the complex. Since we could apply the same approach when using crude bacterial extracts, this also allows analysis of mutant complexes without prior purification.

There are differences amongst the dyes screened with respect to their inhibitory effects on the different subunits, which make them interesting probes. However, the electrophoretic data presented here also show that the dyes tested do not result in the complete separation of a single subunit or a subcomplex. They are, therefore, not suitable for the chromatography purposes that we originally had in mind.

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