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Zone-interference gel electrophoresis: a new method for studying weak protein–nucleic acid complexes under native equilibrium conditions

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ABSTRACT

A new and general electrophoresis method is described for the determination of dissociation constants of weak macromolecular complexes in the range of 10^{-6} to 10^{-4} M. The method is based on the measurement of the migration distance of a macromolecular complex in rapid dynamic equilibrium as a function of the interacting ligand concentration in a surrounding zone. Special advantages of the method are: its high sensitivity (dependent on the autoradiography, immunoblotting or staining technique used), its speed (electrophoresis time 20 min), and the independence of the K_d determination on the sample concentration of macromolecules. The latter is of great value for labile macromolecules: unknown partial inactivation does not influence the measurement. Studying the interactions between elongation factor EF-Tu and tRNA from *E. coli* we found for EF-Tu.GTP.aurodox.aminoacyl-tRNA a K_d of 3 μM and for EF-Tu.GDP.aurodox.aminoacyl-tRNA a K_d of 11 μM at 9 °C.

INTRODUCTION

Retardation gel electrophoresis has become widely used in the study of protein-nucleic acid complexes. The technique is based on the fact that a bound protein retards the migration of the nucleic acid in a non-denaturing gel [1,2]. For a reliable determination of kinetic parameters it is important, that the complexes be relatively long-lived; if the half-life time is too short, no retardation is observed at all. For equilibria with a diffusion controlled association rate this simply means that complexes should have a dissociation constant K_d in the (sub)-nanomolar range. Such values were indeed found for interactions between DNA promoter fragments and activator or repressor proteins. On the other hand, there are numerous examples of more abundantly occurring macromolecules, such as the bacterial elongation factor EF-Tu and its interaction partner aminoacyl-tRNA (cellular concentrations around 100 μM!), where physiologically relevant interactions may take place on the basis of much higher K_d values [3]. The inherently short half-life times of these complexes require equilibrium conditions throughout the analytical procedure to detect any significant interference between the partners at all. In other words, the complex to be analyzed

should migrate continuously through a zone with a constant concentration of ligand molecules.

This principle is used in the classical Hummel-Dreyer gel permeation chromatography [4] or a more sophisticated high-performance liquid chromatography approach [5], and in the so-called sedimentation partition chromatography in sucrose gradients [6]. However, these methods all lack the simplicity, speed, and sensitivity of the retardation gel electrophoresis technique. Here we describe a zone-interference gel electrophoresis procedure that meets all the above-mentioned criteria. The method is illustrated by measurements of interactions between EF-Tu.guanine-nucleotide.aurodox complexes and tRNA, as part of a study in which we are currently engaged [7,8].

THEORETICAL BACKGROUNDS OF ZONE-INTERFERENCE GEL ELECTROPHORESIS

The principle of our method is illustrated in Fig. 1, panels A-D, lane 1. During electrophoresis a complex ML of macromolecule M and ligand L is migrating all the time through a zone with known concentration of L. In this way dissociation is permanently counteracted by association under rapid dynamic equilibrium conditions. After electrophoresis the position of M in the gel is detected by staining, autoradiography or immunoblotting, and the migration distance d_{exp} is measured (Fig. 1, panel D, lane 1). From the measured distances and the concentrations of L in a series of zones, the dissociation constant K_d of the complex ML can be determined as follows. Depending on the value of [L], a certain fraction of time (t_{ML}) the component M is migrating as a complex, while the rest of the time (t_M) it is migrating freely. In a dynamic equilibrium such a time fraction can be described by equation (1).

$$\frac{t_{ML}}{t_{ML} + t_M} = \frac{[ML]}{[ML] + [M]} \qquad (1)$$

Defining d_{ML} as the migration distance of the complex ML, and d_M as that of the free component M, we can characterize d_{exp}, the experimentally observed distance of M, by (2) or, after substitution of (1), by (3).

$$d_{exp} = \frac{t_{ML}}{t_{ML} + t_M} d_{ML} + \frac{t_M}{t_{ML} + t_M} d_M \qquad (2)$$

$$d_{exp} = \frac{[ML]}{[ML] + [M]} d_{ML} + \frac{[M]}{[ML] + [M]} d_M \qquad (3)$$

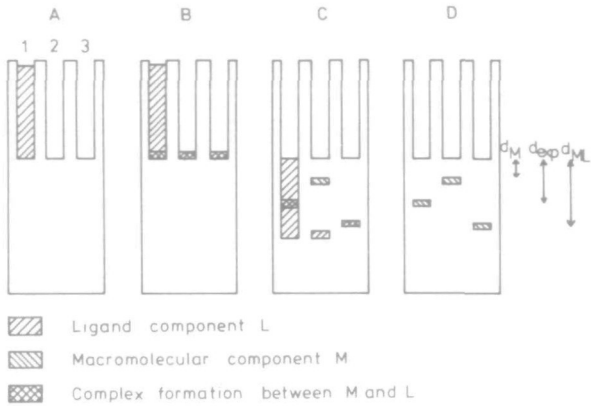

Fig. 1. Schematic comparison of zone-interference gel electrophoresis (lane 1, K_d of complex ML > 10^{-6} M) with gel retardation (lane 3, K_d of complex ML < 10^{-9} M). In lane 2 the same complex is applied as in lane 1, without interfering zone of ligands.
(*A*) Application of zone with known concentrations of ligand L (lane 1). (*B*) Application of samples ML. (*C*) End of electrophoresis. (*D*) Detection of components M. For further details see text.

Elimination of [ML] in (3) by use of the equation $K_d=[M][L]/[ML]$ finally yields (4), in which also [M] has become eliminated.

$$\frac{d_{exp} - d_M}{[L]} = -\frac{d_{exp} - d_{ML}}{K_d} \qquad (4)$$

This is a Scatchard-like formula: when $(d_{exp}-d_M)/[L]$ is plotted against d_{exp}, a straight line results with a slope of $-1/K_d$ and an intercept of d_{ML} at the horizontal axis (compare Fig. 6).

The ambient concentration of L at the band containing M and ML remains constant throughout complex migration irrespective of the concentration of M: in the dynamic equilibrium in the migrating band there is at the trailing edge a constant inflow of molecules of L at constant concentration, compensating for the constant outflow of non-complexed L at the leading edge of the band. In order to approximate this situation from the very beginning of the electrophoresis, the sample is prepared by preincubating a tiny amount of M plus the equivalent amount of L in a solution with the desired zone-concentration [L]. Thus, even here the amount of L is not limiting and, since the technique has been developed for fast dissociating complexes, soon after

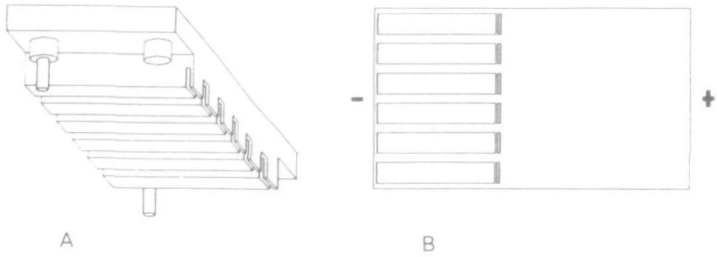

Fig. 2. Requisites for the horizontal open gel system.
(*A*) Comb for the casting of zone slots (22.5x4x2.5 mm) and sample slots (1x4x2.5 mm). (*B*) Schematic upper view of the agarose gel with zone and sample slots and with the electrode positions indicated.

starting the separation the equilibrium reaches the proper situation as described in (4).

MATERIALS AND METHODS

Guanine nucleotides, phospho*enol*pyruvate, pyruvate kinase and bulk tRNA from *E. coli* MRE 600 were purchased from Boehringer Mannheim, FRG. Agarose was from Bethesda Research Laboratories, Neu Isenburg, FRG. Kirromycin and aurodox (N^1-methyl kirromycin) were gifts from Gist-Brocades, Delft, The Netherlands; their effects on EF-Tu were indistinguishable. The preparation of EF-Tu from *E. coli* MRE600, the aminoacylation of bulk tRNA and complex formation between the two were described previously [8]. The completeness of the aminoacylation reaction was checked with a mixture of [^{14}C]-aminoacids obtained from NEN Research Products, Du Pont de Nemours, Dreieich, FRG. A thermosensor (0.5 mm diameter, ISA-K) was purchased from Thermo Electric, Warmond, The Netherlands.

Electrophoresis Procedures. Zone-interference gel electrophoresis can be performed with two kinds of systems. Horizontal gel electrophoresis with a mini-gel system can be used for quick determinations, vertical gel electrophoresis is appropriate for more extensive and accurate measurements under controlled cooling conditions.

For horizontal electrophoresis, a warmed 2% (w/v) solution of agarose in electrophoresis buffer (20 mM Tris acetate pH 7.6, 3.5 mM Mg acetate) is poured into the bed (75x50x3 mm) of a mini-gel apparatus. Immediately, a comb with 6 zone and sample slots (Fig. 2A) is placed in the agarose and the rest of the bed is covered by a glass plate to obtain a gel with even thickness and to facilitate the removal of the comb without lifting up the gel. In the

next step, a series of zone solutions (200 µl) of increasing ligand concentrations in electrophoresis buffer with 10% (v/v) dimethylsulfoxide and a trace of bromophenol blue is pipetted into the extended zone slots on the cathode side of the gel (Fig. 2B). For about a minute, a voltage of 150 V is applied until the marker dye has arrived in the sample slots. Then, the sample solutions (see the paragraph on Theoretical Backgrounds) in 10 µl of the same buffer with 10% (v/v) dimethylsulfoxide are applied. Thereafter, the gel is covered with a 5 mm layer of n-pentane for cooling by evaporation and the gel is run in a fume cupboard at 150 V for 20 min. After electrophoresis the gel is cut off at the dye front and fixed in 3.5% (w/v) α-sulfosalicylic acid, 10% (w/v) trichloroacetic acid until the dye turns yellow. At this stage, zones with a high concentration of ligands such as tRNA can show up as a white opalescent area. For the detection of protein bands (in our case EF-Tu), the gel is washed for 15 min in 15% (v/v) ethanol, 8% (v/v) acetic acid, and stained for 30 min with 0.25% (w/v) Coomassie Brilliant Blue in the same solution containing an additional 10% (v/v) methanol. The gel is washed in 15% (v/v) ethanol, 8% (v/v) acetic acid and stored in 10% (v/v) acetic acid. Alternatively, RNA bands may migrate as "M" in zones of protein as "L". In such a case, the gel is transferred from the fixation medium to a solution of 0.2% (w/v) methylene blue in 0.2 M K-acetate pH 4 for 1 h, and destained with a solution of 50% (v/v) methanol, 5% (w/v) glycerol.

Vertical electrophoresis is performed in a gel (150x150x2 mm) with 20 extra deep slots (28x4x2 mm), by use of the same gel and buffer conditions as described above. Cooling is here effected by means of hollow front and backplates through which ice-cold water is run (Fig. 3). The gel temperature can be monitored by a needle shaped thermosensor in the gel itself or estimated by a thermometer in the water outlet.

After gel casting, one of the plates is taken off, the comb is cut out of the gel, the plate is replaced and the bottom side sealed with 10% (w/v) acrylamide, 0.2% (w/v) bisacrylamide in electrophoresis buffer. In the next step, a similar series of zone solutions (200 µl) and sample solutions (10 µl) is applied, in such a way that the former (containing 4% (v/v) DMSO) is pipetted first and the latter (containing 10% (v/v) DMSO and the marker dye) is layered underneath. Electrophoresis takes place at 400 V for 20 min. The gel is stained as described above or blotted on nitrocellulose for autoradiography or immunodetection [9,10]. In the latter case, methyl green can be used as an additional marker in the zone solution. During the run methyl green hardly migrates. It diffuses into the agarose and, after

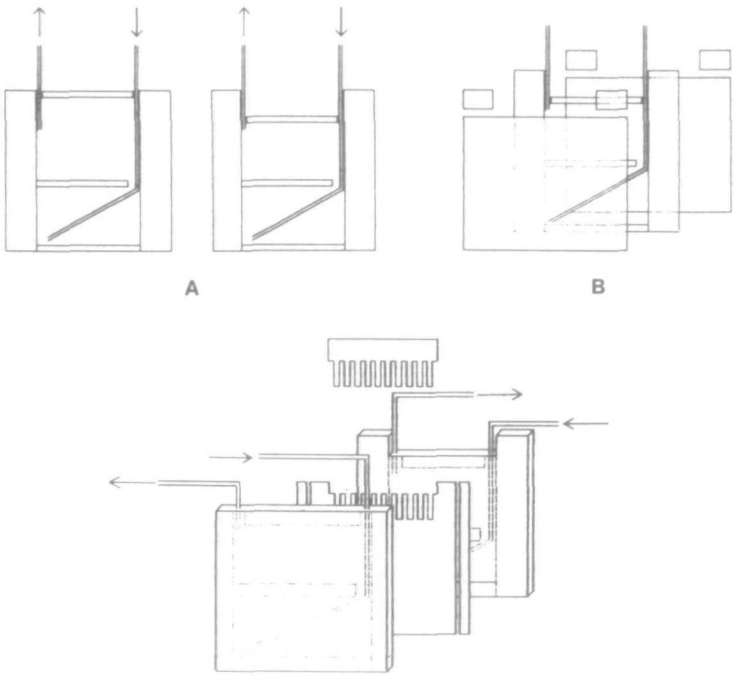

Fig. 3. Requisites for the vertical system.
(*A*) Front views of hollow front and back cooling plates between which the agarose gel is cast with 2 mm spacers. Cooling water in- and outlets are indicated. (*B*) Exploded view of the backplate construction. The cover plate sections are cut from 2 mm glass plate, the spacer sections are from 4 mm glass plate. Glass pieces and teflon tubing (3.5 mm diameter) are glued together with silicone kit. (*C*) Exploded view of the assembled system with front and back plates, gel, comb and gel spacers. For simplicity only 10 slots are drawn.

blotting it leaves a colour print of the slots on the nitrocellulose, thus facilitating the measurement of d_{exp}.

RESULTS AND DISCUSSION

A few examples of zone-interference gel patterns of aurodox containing EF-Tu complexes in interaction with tRNA are shown in Fig. 5. Under normal cellular conditions EF-Tu.GTP but not EF-Tu.GDP forms ternary complexes with aminoacyl-tRNA. The binding of aurodox or kirromycin to EF-Tu.GDP, however, activates the binding site for aminoacyl-tRNA with reported K_d values of 0.9×10^{-6}M (6°C) and 3×10^{-6}M (25°C) for Phe-$tRNA_{E.\ coli}$ [12] and Phe-$tRNA_{yeast}$ [13], respectively. Both antibiotics (Fig. 4) are potent inhibitors

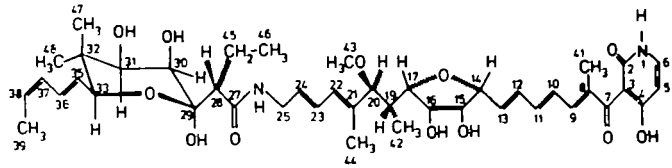

Fig. 4. Structure of the antibiotic kirromycin. The N^1-methyl derivative is called aurodox.

of protein biosynthesis by immobilizing EF-Tu.GTP/GDP.aminoacyl-tRNA complexes on ribosomes (for a review see [11]). Figure 5B shows indeed a considerable mobility increase of the EF-Tu band upon raising the aminoacyl-tRNA concentrations in the zones. Such an increase is not observed with deacylated tRNA (Fig. 5A), which underlines the specificity of the interactions of Fig. 5B. Another specificity control comes from a similar experiment with ovalbumin or serum albumin instead of EF-Tu. Neither of them displays an altered mobility in the presence of high aminoacyl-tRNA concentrations (results not shown).

We would like to stress here, that the interactions of Fig. 5B would indeed have escaped detection via retardation gel electrophoresis. This is evident from the EF-Tu migration in the zone with [aminoacyl-tRNA]=0 (compare also Fig. 1, lane 2). Whereas in the sample equimolar amounts (4 μM) of EF-Tu.GDP.aurodox and aminoacyl-tRNA are present, a rapid dissociation of the formed complexes occurs from the onset of the electrophoresis and no significant effect on the migration distance of the EF-Tu band can be observed.

In Fig. 5C and D similar experiments are shown as in A and B, but now EF-Tu.GTP.aurodox is the partner in complexes with tRNA. Again, deacylated tRNA does not show a significant interaction in the concentration range studied. With aminoacyl-tRNA, however, the formation of two different complexes can be observed.

From migration data such as shown in Fig. 5B and D plots can be made in order to calculate the K_d value. This is illustrated in Fig. 6A and B, respectively. The complex EF-Tu.GDP.aurodox.aminoacyl-tRNA appears to have a K_d of 11 μM. This is an average value for a mixture of all the tRNA species together and is in the same order of magnitude as the 3 μM mentioned above for $Phe\text{-}tRNA_{yeast}$. From Fig. 5D two curves were constructed in Fig. 6B with corresponding K_d values of 3 μM and 10 μM for EF-Tu.GTP.aurodox-aminoacyl-tRNA and EF-Tu.GDP.aurodox.aminoacyl-tRNA, respectively. The explanation is

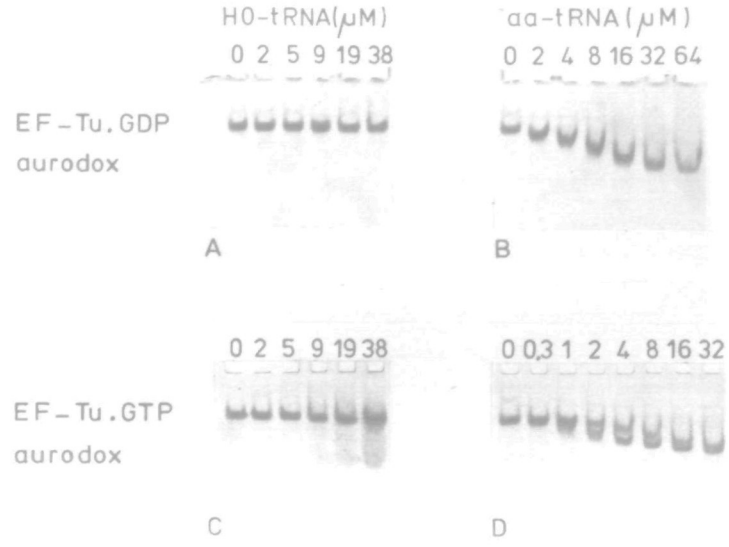

Fig. 5. Zone-interference gel electrophoresis of mixtures of various EF-Tu complexes and tRNA preparations in the vertical gel system. Samples containing complexes of 4 μM EF-Tu.GDP.aurodox mixed with 4 μM deacylated tRNA (*A*) or aminoacyl-tRNA (*B*), and complexes of 4 μM EF-Tu.GTP.aurodox mixed with 4 μM deacylated tRNA (*C*) or aminoacyl-tRNA (*D*) were applied in zones containing either deacylated tRNA or aminoacyl-tRNA in the concentrations indicated. In addition to the standard components, the gel and electrophoresis buffer contained 1 μM aurodox and either 10 μM GDP (in *A,B*) or 10 μM GTP (in *C,D*). During electrophoresis the gel temperature was about 9°C. Protein bands were stained with Coomassie Brilliant Blue. For further details see Materials and Methods.

that aurodox and kirromycin activate the GTPase center of EF-Tu, an effect that is further enhanced by aminoacyl-tRNA binding [11,8]. For the complex EF-Tu.GTP.aurodox.aminoacyl-tRNA, well-documented literature values were not yet available. Our results, however, clearly show that an earlier estimate of about 0.3 μM [13] must have been a bit too low.

An important reason for us to develop the present method came from previous experiments [8] which pointed to the formation of EF-Tu.kirromycin complexes with two simultaneously bound tRNA molecules. Such complexes could not be found in the present study. We cannot exclude the possibility, however, that EF-Tu complexes with one and two tRNA molecules may not differ much in electrophoretic mobility, the effect of the higher negative charge of the latter complex being compensated by a larger Stokes' radius. Further details about other EF-Tu-tRNA interactions wil be published elsewhere.

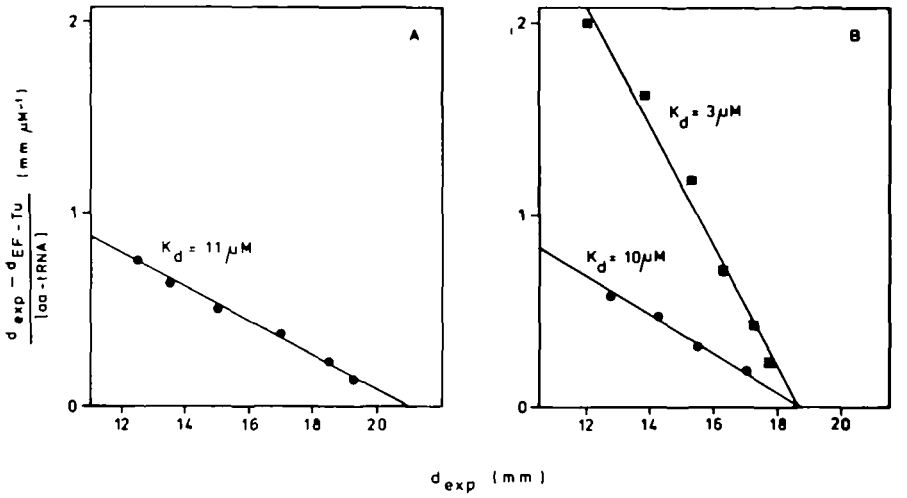

Fig. 6. Graphical calculation of K_d values from the experimental data in Fig. 5, according to equation (4) (see Theoretical Backgrounds). In (*A*) the data from Fig. 5B for the equilibrium between EF-Tu.GDP.aurodox and aminoacyl-tRNA are plotted. In (*B*), the data from Fig. 5D are plotted, corresponding to EF-Tu.GTP.aurodox.aminoacyl-tRNA (■) together with EF-Tu.GDP.aurodox.aminoacyl-tRNA (●).

Finally, we would like to mention the advantages and limitations of the present method. Actually, it has some aspects in common with equilibrium dialysis, without the physical barrier of a dialysis membrane. Just like all the gelelectrophoretic techniques it is highly sensitive (dependent on the autoradiography, immunoblotting or staining techniques used) in comparison with column or ultracentrifuge techniques.

Due to the large pore size of the agarose gel and the low ionic strength of the buffer, the electrophoresis takes little time (important for labile components like aminoacyl-tRNAs). It is performed under controlled temperature conditions. As appears from formula (4), determination of the K_d is independent of the concentration of active macromolecules in the sample; it is only the ligand concentration in the set of zones that counts. This is a great advantage for labile proteins; the ever present uncertainty about the extent of denaturation is overcome. The K_d can even be directly estimated from the gel pattern without plotting: it equals the [L] value of the zone in which d_{exp} is just in the middle between d_M and estimated d_{ML} (compare formula (4)). Several complexes may be analyzed together in the same sample

(compare Fig. 5D) and even in crude extracts a K_d could be determined if a specific detection method (*e.g.* immunoblotting, ^{32}P-probe hybridization) for the complex of interest is available. An obvious requirement of the present method is that the complex and its constituents all move in the same direction but do not comigrate, similar to the requirement of the gel retardation method [14]. In the above-mentioned examples the zones with L move faster than the samples with M. In other cases one can easily adapt the sample application procedure: in a preelectrophoresis step the ligand zone is almost entirely transferred into the gel, whereafter the sample is applied and the actual zone-interference electrophoresis can start.

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