*Journal of Chromatography*, 449 (1988) 331–336
Elsevier Science Publishers B.V., Amsterdam — Printed in The Netherlands

CHROM. 20 702

## Note

# Detection of ligand–protein binding by direct electrophoresis of the complex

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(First received March 22nd, 1988; revised manuscript received June 6th, 1988)

The detection of binding of a ligand to a protein is an analytical process usually accomplished by equilibrium dialysis[1], ultrafiltration[2] or, in the case of an uncharged ligand, by isoelectric focusing in the presence of the radioactive ligand followed by autoradiography[3]. Other techniques such as precipitation of the complex by ammonium sulphate[4] or fixation of the complex on nitrocellulose membranes[5] have been successful in some cases, but do not allow the discrimination of the binding protein from the other unrelated proteins present in the extract. The equilibrium dialysis or ligand retention dialysis methods[6] are interesting since they allow the determination of the affinity constant of the protein for its ligand. However, these methods have the disadvantage of using large amounts of proteic material. Binding is the ratio between the rates of association and dissociation. For bacterial binding proteins, the rate of association has been reported to be almost diffusion controlled, while dissociation was slow but independent of association[7]. It is believed that isoelectric focusing or electrophoresis techniques cannot be used in the presence of charged ligands, which would dissociate and be eliminated during the analysis.

In this paper we show that complexes of radioactive ligands with bacterial periplasmic binding proteins, such as the γ-butyrobetaine-binding protein, leucine-binding protein and ribose-binding protein, are sufficiently stable to be detected by autoradiography after non-denaturing polyacrylamide gel electrophoresis (PAGE) using a minigel system. Thus, binding proteins can be specifically identified by their relative positions on polyacrylamide gels.

## EXPERIMENTAL

### *Bacterial strains and growth conditions*

*Agrobacterium* sp. strain $HK_4$ (DSM2938) was grown at 30°C with γ-butyrobetaine as a carbon and nitrogen source and to induce the transport system[8]. Non-induced cells were grown on 0.2% D-glucose. Strains AI271 and W1485 are derivatives of *Escherichia coli* K-12 and were grown at 37°C. Strain AI271[9] was grown on M9 minimum salt medium with 1% glycerol and 0.2% ribose as carbon sources. Strain W1485 was grown on minimal medium supplemented with 1% glycerol as a carbon source.

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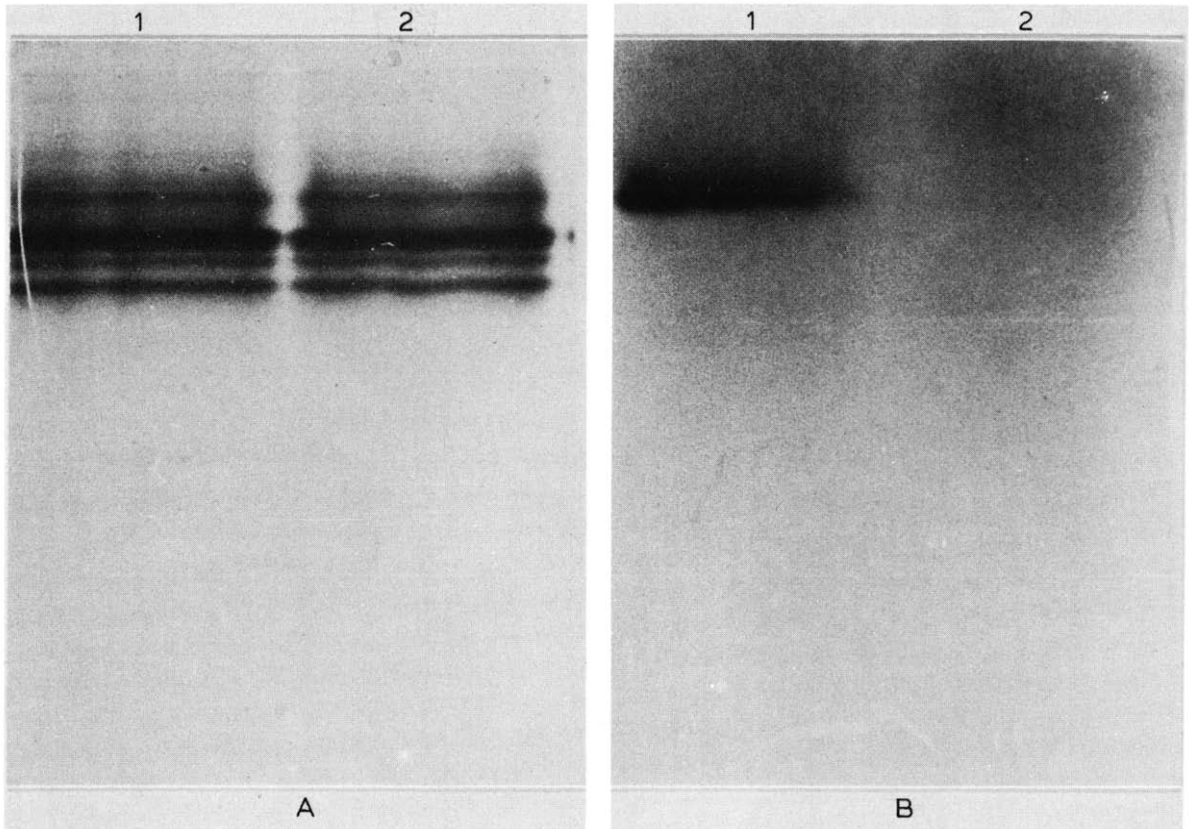


Fig. 1. 12% Non-denaturing PAGE (A) and autoradiography (B) of periplasmic proteins obtained from *E. coli* strain AI271. The periplasmic extracts (50 $\mu g$) were incubated for 15 min with 10 $\mu M$ [$^{14}$C]D-ribose in the absence (lane 1) or presence (lane 2) of 500 $\mu M$ unlabelled D-ribose.

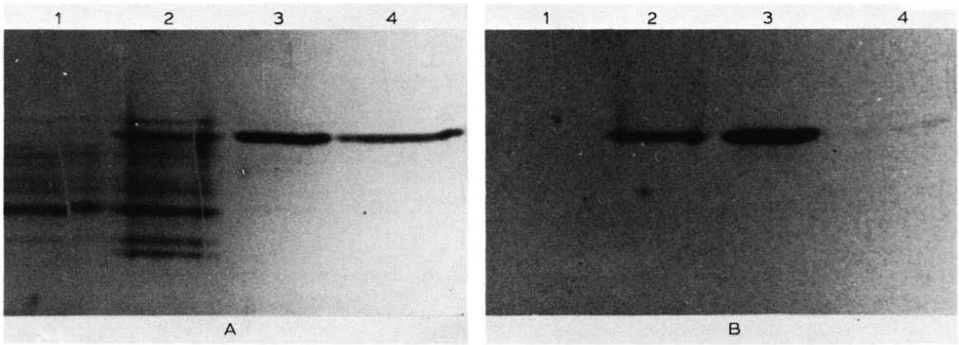


Fig. 2. 10% Non-denaturing PAGE (A) and autoradiography (B) of periplasmic proteins from non-induced *Agrobacterium sp.* HK4 cells (lane 1), periplasmic proteins from induced *Agrobacterium sp.* HK4 cells (lane 2), or purified γ-butyrobetaine-binding protein (lanes 3 and 4). A 50-μg amount of the proteins was incubated for 15 min with 10 μ*M* [$^{14}$C]γ-butyrobetaine in the absence (lanes 1, 2 and 3) or presence (lane 4) of 500 μ*M* unlabelled γ-butyrobetaine.

*Osmotic shocks*

Osmotic shocks were performed according to Neu and Heppel[10]. The periplasmic protein extracts were filtered on membranes (Sartorius, Göttingen, F.R.G.), concentrated by ultrafiltration on YM10 Amicon membranes (Diaflo Amicon, Oosterhout, The Netherlands) to a final concentration of approximately 1 mg/ml and extensively dialyzed against 10 m*M* Tris–HCl buffer, pH 7.4.

*Electrophoresis and autoradiography*

Aliquots of the concentrated shock fluids containing the various binding proteins (50 μg) were mixed with the respective radioactive ligands [$^{14}$C]γ-butyrobetaine (16 μCi/μmol), [$^{14}$C]L-leucine (330 μCi/μmol) and [$^{14}$C]D-ribose (58 μCi/μmol), at a final concentration of approximately 10 μ*M* and left at room temperature for 15 min. Laemmli's sample buffer[11] (without sodium dodecyl sulphate and β-mercaptoethanol) was then added and the samples were subjected, without denaturation and reduction, to PAGE in a discontinuous system in which sodium dodecyl sulphate was omitted. In order to shorten the time of electrophoresis, the Bio-Rad mini protean™ slab cell system (Bio-Rad Labs., Richmond, CA, U.S.A.) was used. The gels were 6 cm long, including a stacking of approximatively 1 cm and the spacers were 0.75 mm thick. The analyses were performed with a constant voltage setting of 200 V, usually for approximately 45 min. The gels were then quickly dried on Whatman Nos 3MM paper and autoradiographed using Fuji X-medical films (Fuji Photo Film, Tokyo, Japan). Coomassie blue staining was performed after autoradiography by scraping the Whatman paper from the gel with ethanol.

*Protein determination*

Protein concentrations were determined according to Bradford[12].

*Chemicals*

[$^{14}$C]L-Leucine and [$^{14}$C]D-ribose were obtained from the Radiochemical Centre (Amersham, U.K.). [$^{14}$C]γ-Butyrobetaine was prepared in our laboratory by methyla-

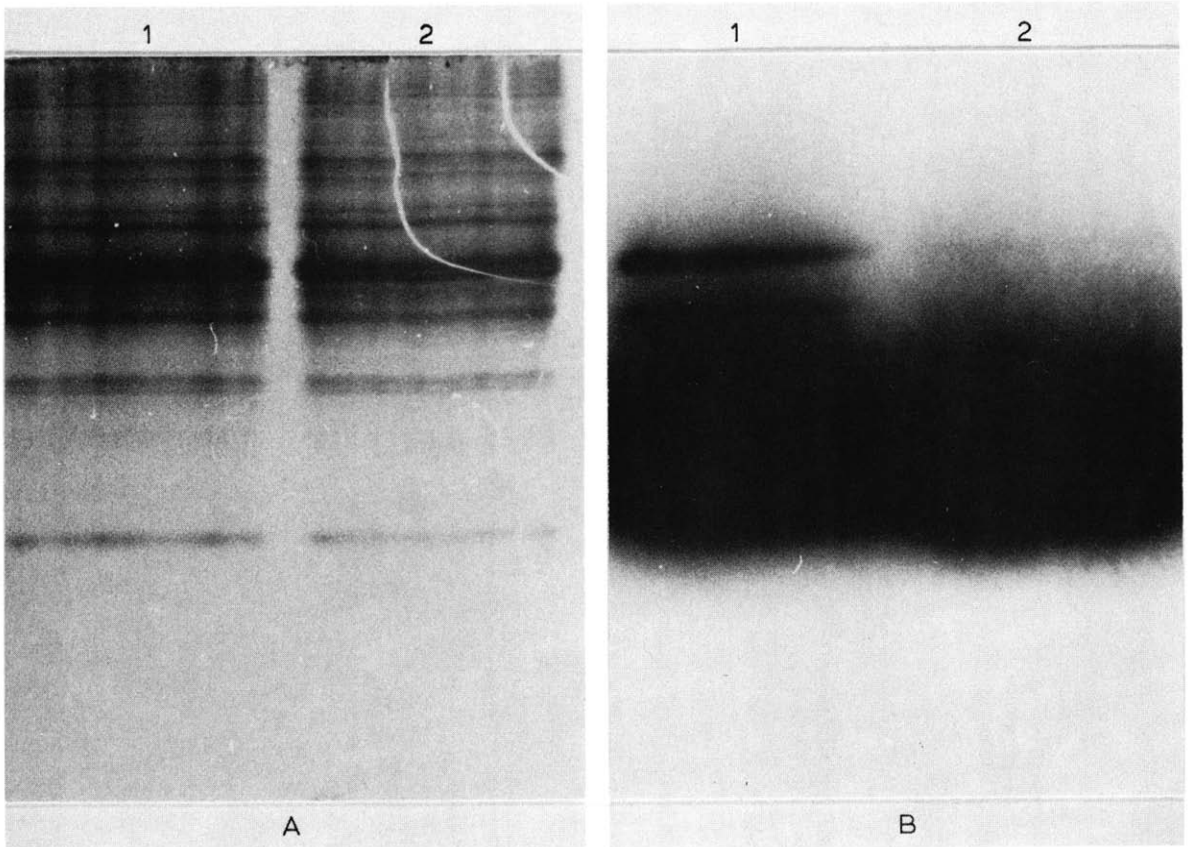


Fig. 3. 12% Non-denaturing PAGE (A) and autoradiography (B) of periplasmic proteins obtained from *E. coli* strain W1485. The periplasmic extracts (50 $\mu$g) were incubated for 15 min with 10 $\mu M$ [$^{14}$C]L-leucine in the absence (lane 1) or presence (lane 2) of 500 $\mu M$ unlabelled L-leucine.

tion of [$^{14}$C]γ-aminobutyric acid (Radiochemical Centre). The other chemicals were of the highest purity available from E. Merck (Darmstadt, F.R.G.), or Fluka (Buchs, Switzerland).

## RESULTS AND DISCUSSION

In order to show that electrophoresis of complexes is a convenient method to detect the presence of a specific binding activity, crude shock fluids of bacteria induced for three different specific transport systems were used. For the detection of ribose-binding proteins, on the gel shown in Fig. 1 only one band is labelled within the mixture of the shock released proteins (lane 1). This result is in accord with the literature, indicating the involvement of one ribose-binding protein in *E. coli*[13]. When unlabelled D-ribose (500 $\mu M$) was added to the mixture a significant decrease in the labelling was observed, demonstrating the specificity of the binding phenomenon (lane 2).

Crude shock fluid obtained from an *Agrobacterium* sp. isolated from soil and able to concentrate γ-butyrobetaine (an highly charged molecule) through an inducible binding protein-dependent transport system[8] was subjected to the same procedure (Fig. 2, lane 2). Also shown is a protein preparation from non-induced cells grown on glucose (lane 1), and two samples of the purified γ-butyrobetaine-binding protein (lanes 3 and 4). With the crude extract as well as with the pure protein (lanes 2 and 3), a radioactive band was found at the same position, showing the presence of the label bound to the binding protein. The fact that only the γ-butyrobetaine-binding protein was labelled in the crude extract rules out the possibility of a non-specific binding. Finally, addition of unlabelled γ-butyrobetaine (500 $\mu M$) in the purified preparation (lane 4) resulted in a nearly complete abolition of the labelling.

L-Leucine is transported in *E. coli* by two distinct transport systems each involving distinct binding proteins (LIV-binding protein and LS-binding protein)[14]. Although these proteins have very similar molecular masses[13], they were distinguished on our gel system despite the broad radioactive band caused by the free ligand (Fig. 3, lane 1). Here again, the addition of non-radioactive L-leucine resulted in a significant decrease in the protein labelling (lane 2).

In conclusion, we have shown that non-denaturating PAGE using a minigel system allows the detection of various bacterial binding proteins specific for neutral or highly charged molecules such as amino acids or betaines. The simplicity of the procedure should help in the detection and the purification of various proteins involved in binding phenomena.

## ACKNOWLEDGEMENTS

Financial support was provided by a grant from Lonza (Basel, Switzerland). We thank A. Iida and L. Caro for strain AI271 and W1485 respectively, M. Belet for performing the osmotic shocks and L. Poljak for her critical reading of the manuscript.

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