Renaturation and Identification of Periplasmic Proteins in Two-dimensional Gels of *Escherichia coli*

(Received for publication, March 29, 1982)

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The locations of the periplasmic proteins of *Escherichia coli* on standard two-dimensional gel patterns are described. The periplasmic fractions were prepared by osmotic shock of plasmolyzed whole cells and by release during EDTA-lysozyme treatment of whole cells. Within this fraction of proteins, we identify nine binding proteins (leucine-specific, glutamate-aspartate, glutamine, cystine, galactose, maltose, xylose, ribose, and arabinose) in addition to leucine-isoleucine-valine binding protein, which has been previously identified (Bloch, F. L., Phillips, T. A., and Neidhardt, F. C. (1980) *J. Bacteriol.* 141, 1409–1420). The identifications are based upon genetic criteria, protein induction, and comigration with purified protein. The levels of these proteins are compared in strains K12, B, and HA12 (a derivative of W). A technique was developed for renaturation of the ligand binding sites of periplasmic binding proteins in denaturing two-dimensional gels. This technique was used to demonstrate that leucine-specific and cystine binding proteins both have different isoelectric points in different strains. Renaturation was also used to demonstrate that there are two different charged forms for glutamine binding protein.

Two-dimensional gel electrophoresis (1, 2) is a powerful method that is capable of resolving complex mixtures of proteins even in very small amounts. A single gel can therefore provide large amounts of diverse information about the physiology and genetic composition of a sample of cells, provided that many of the proteins on the gel are identified.

Recently a sizable number of *Escherichia coli* proteins have been identified by Bloch et al. (3) and Phillips et al. (4) on standardized two-dimensional gel patterns. Nevertheless, very few transport components or periplasmic proteins are represented in the proteins identified on these patterns. Our laboratory has begun a general program to locate the proteins from different subcellular fractions of *E. coli* on the standardized patterns of whole cell proteins. As part of this program, we report here the locations of periplasmic proteins on whole cell patterns for *E. coli*, and we identify ten specific periplasmic binding proteins that are involved in amino acid and sugar transport. We compare the levels and locations of these binding proteins in representatives of three major *E. coli* strains. In addition, we describe a method for renaturing the binding sites of periplasmic proteins in denaturing two-dimensional gels. This renaturation technique is used to identify several of the binding proteins in different strains, and should be useful for identification of virtually any protein in which a high affinity binding site can be renatured.

**Materials and Methods**

**Strains and Growth Conditions—**Wild type *E. coli* B was obtained from Professor A. Doermann (University of Washington). Wild type K12 strain was obtained from Professor L. Heppel (Cornell University). Strain HA12-GA7 (an overproducer for glutamate-aspartate binding protein) and its parent strain HA12 were derived previously by several mutations from strain W (5, 6).

Except where noted, all cells were grown on the minimal MOPS medium of Neidhardt et al. (7), supplemented with appropriate carbon sources. Carbon-limited starter cultures were grown overnight with 0.04% (v:v) glycerol. These cultures were then diluted into 10 ml of minimal MOPS medium containing 1% (v:v) glycerol and 1% (w:v) of appropriate sugars for induction. Cells were grown in the latter media at 37 °C with vigorous aeration for at least four generations, and were harvested when the cultures reached an absorbance of 1 to 1.5 at 420 nm (A420). This corresponds roughly to 5 × 10⁸ cells/ml.

**Preparation of Periplasmic Fractions—**Osmotic shock was performed using a small scale modification of the method of Willis et al. (8). Cultures containing a total of 10 A₄₂₀ units were preconditioned for shock by the addition of 0.03 volume each of 1 M NaCl and 1 M Tris-HCl buffer, pH 7.3. Cells were harvested (3000 × g, 10 min, 4 °C) and resuspended in 50 ml of MOPS medium containing 1% glycerol and 0.03 volume of 1 M NaCl and 1 M Tris-HCl buffer, pH 7.3. The resuspended cells were centrifuged for 1.5 min in a Microfuge at room temperature. The resulting pellet was suspended in 50 μl of 0.03 M Tris-HCl buffer, pH 7.3. To this suspension were added 50 μl of 40% (w:v) sucrose in 0.03 M Tris-HCl buffer, 2 mM EDTA, pH 7.3. The cells were plasmolyzed for 15 min at room temperature, then centrifuged for 1.5 min in the Microfuge. The supernatant was removed. The cells were next mixed vigorously with 100 μl of cold, distilled water for 30 s. After an additional 15 s on ice, 5 μl of 20 mM MgCl₂ were added, and the suspension was mixed for 10 s. The timing on these steps was critical for a reproducible osmotic shock. The suspension was allowed to stand on ice for several minutes before centrifuging in the Microfuge for 3 min. The supernatant containing the periplasmic proteins was carefully removed and centrifuged again for 3 min in the Microfuge to remove any contaminating cells.

Spheroplasting of cells by EDTA-lysozyme was performed as follows. Cells from 35 A₄₂₀ units of culture were harvested (16,000 × g, 10 min, 4 °C) and washed once in 10 ml of 30 mM Tris-HCl buffer, pH 8.0. The pellet was resuspended in 350 μl of 20% (w:v) sucrose in 30 mM Tris-HCl buffer, pH 8.0, warmed to 37 °C. A freshly prepared solution of 5.0 mg/ml of lysozyme in 100 mM EDTA was then added 50 μl into the suspension of cells at 37 °C. The extent of sphero-
absorbance at 600 nm ($A_{600}$) for 1:30 dilutions of cell suspension into distilled water. After 10 min of incubation at 37 °C, less than 5% of cells were rods, and the $A_{600}$ for diluted cell suspension had dropped from 1.15 to 0.09. After an additional 10 min of incubation, the spheroplasting was judged complete. The suspension was centrifuged for 9 min in the Microfuge, and the supernatant containing periplasmic proteins was removed and centrifuged again for 9 min to remove contaminating cells. The final supernatant was diluted 1:1 with distilled H2O.

**Purified Binding Proteins**—Arabinose binding protein (9) from strain B/r was kindly provided by Dr. W. Mahoney (University of Washington). All other binding proteins were from frozen stocks of proteins prepared in this laboratory. Leucine-isoleucine-valine binding protein (10), leucine-specific binding protein (11), galactose binding protein (12), cystine-binding protein (13), and cystine binding protein (14) were from K12 sources. Glutamate-aspartate binding protein (15) and glutamine binding protein (16) were from HAI2-G7.

**Two-dimensional Gel Electrophoresis**—Samples for two-dimensional gel electrophoresis were prepared using the solutions and methods of O'Farrell (1). For whole cell samples, 2.5 $A_{600}$ units of cells were harvested (3000 × g, 10 min, 4 °C) and resuspended in 500 µl of medium. The suspension was then centrifuged for 1.5 min in a Microfuge at room temperature. The pellet was resuspended in 100 µl of sonication buffer (1) and sonicated on ice using a Heat Systems Usonicifier equipped with a microprobe tip. Three 30-s bursts of power were alternated with 15-s periods of cooling. DNase was then added to a level of 50 µg/ml, and the suspension was incubated on ice for 15 min. Finally, an equal weight of urea was dissolved, and the resulting volume was mixed 1:1 with O'Farrell lysis buffer. Samples of shocked cells were prepared in the same way by sonicating shocked cells from 2.5 $A_{600}$ units of culture. Periplasmic fractions were treated with RNase (50 µg/ml) on ice for 15 min, followed by the addition of 2-mercaptoethanol to 1 mM. Samples were prepared by the addition of an equal weight of urea and dilution with lysis buffer exactly as described for whole cells. All samples were stored at −70 °C.

The methods for two-dimensional gel electrophoresis were as previously described (1, 2, 17), with the following special conditions. Equilibrium isoelectric focusing of samples was performed for 14.5 h at 34 V/cm, followed by 1 h at 68 V/cm. Nonequilibrium isoelectric focusing was performed for 3.75 h at 34 V/cm. Focused gels were equilibrated for 40 min in sodium dodecyl sulfate sample buffer before storage at −70 °C or electrophoresis on second dimension gels. Resolving gels in the second dimension contained 12% (w:v) acrylamide; stacking gels were 2 cm in height.

Gels were silver-stained using modifications of the procedure of Oakley et al. (18) as previously described (17). The fixation reagent was further modified to contain 2.5% formaldehyde (prepared by boiling 2.5% (w:v) p-formaldehyde) in addition to 2.5% glutaraldehyde. The formaldehyde appears to improve staining of low molecular weight proteins and decreases background in the stained gels. It was therefore unnecessary to use photoreducer to lighten background in the stained gels.

**Renaturation of Binding Proteins in Gels**—Two-dimensional gels of osmotic shock fluid were run as usual, except for two modifications. Shock fluid was concentrated as much as 10-fold by lyophilization before sample preparation, and 2-mercaptoethanol was omitted from all sample and electrophoresis solutions. The resulting gels contained much protein, and there was some distortion in the patterns, but the patterns were not substantially different from standard patterns (cf. Figs. 1b and 5e). After electrophoresis, a section of gel (6 × 3 cm) that contained the gel region of interest was cut from the remainder of the two-dimensional gel. This section was transferred to a plastic tissue culture flask of dimensions 6.5 × 3.5 × 2 cm. It was rinsed three times for 10 min each in 10 ml of 50 mM Tris-HCl buffer, pH 7.3, containing 10% (w:v) glycerol (R buffer). It was then incubated for 15 min in 2.5 ml of R buffer containing 2.5 µCi of the appropriate 3H-aminoacid or sugar at approximately 3 µM ligand concentration. Subsequently, the gel section was rinsed twice for 10 min each in 10 ml of R buffer. It was then briefly rinsed in distilled water and dried onto filter paper (37 °C, 2 h) under vacuum. Kodak X-OMAT AR film was exposed to the dried gel for 75 to 300 h at room temperature. After autoradiography, the gel was rehydrated in 50% (v:v) methanol and 12% (v:v) acetic acid for 30 min, and the filter paper backing was removed. (Alternatively, some gel sections were immersed in 90% (v:v) methanol and then gradually diluted to 50% methanol and 12% acetic acid.) The gel section was then swelled in several changes of 10% (v:v) ethanol and 5% (v:v) acetic acid over a 1.5-h period. Finally it was rinsed three times for 10 min in 10% (v:v) ethanol before silver staining with ¼ the volumes used in the usual full size gel procedure.

**RESULTS**

Fig. 1 shows the equilibrium isoelectric focusing two-dimensional gel patterns for whole cell proteins and osmotic shock fluid proteins of *E. coli* strain B. The whole cell gel pattern is substantially similar to the standardized pattern published by Bloch et al. (3). There are a few differences due to growth on glycerol as a carbon source instead of glucose, which was used by Bloch et al. There are also some differences that apparently result from subtle variations in electrophoretic conditions and storage at -70 °C or electrophoresis on second dimension gels. Resolving gels in the second dimension contained 12% (w:v) acrylamide; stacking gels were 2 cm in height.

![Fig. 1. Equilibrium (pH 5–7) focusing two-dimensional gels of strain B proteins. a, whole cell proteins from 0.19 $A_{600}$ unit of culture grown on glycerol; b, osmotic shock fluid proteins from 0.76 $A_{600}$ unit of culture grown on glycerol. Sample preparation and electrophoresis were as described under "Materials and Methods." The rectangles in a indicate the locations of pancreatic DNase that was added during sample preparation. Approximate molecular weights correspond to *E. coli* β-galactosidase, rabbit phosphorylase b, bovine serum albumin, ovalbumin, bovine carbonic anhydrase, soybean trypsin inhibitor, and horse heart cytochrome c, which were run in parallel gels as described in Ref. 17.](image-url)
our use of silver stain for visualization instead of autoradiography. Visualization by this particular silver stain requires larger loads of protein than autoradiography, and some distortion in the pattern results. However, the silver stain gives

**Fig. 2. Locations of periplasmic proteins on two-dimensional gel position patterns for whole cells of strain B grown on glycerol.** a, equilibrium (pH 5-7) focusing pattern; b, nonequilibrium focusing pattern. Periplasmic proteins are indicated in gray and nonperiplasmic proteins are indicated in white in a and to the left of the dashed line in b. To the right of the dashed line in b, the actual negative image of the gel is shown for reference; resolution is poor in this region of the pattern, and position information is conveyed more effectively for this pH region in a. Designations of periplasmic proteins are based upon criteria described in the text. Molecular weight information is as for Fig. 1. Specific protein identifications are indicated by arrows. The methods used for identification are outlined in Table I. Identifications that are based on the locations of proteins identified at corresponding gel positions for another strain are indicated in parentheses. Arabinose BP and xylose BP do not appear in detectable amounts on gels of cells grown in glycerol. The positions are indicated where these proteins would appear if present. Only the more acidic of the two spots corresponding to glutamine (GlnBP) appears reproducibly on equilibrium (pH 5-7) patterns. RBP, ribose BP; LIVBP, leucine-isoleucine-valine BP; GBP, galactose BP; LeuBP, leucine BP; XBP, xylene BP; ABP, arabinose BP; GABP, glutamate-aspartate BP.

**Fig. 3. Locations of periplasmic proteins on two-dimensional gel position patterns for whole cells of strain K12 grown on glycerol.** Notation is as in Fig. 2. Designations of periplasmic proteins for K12 are based upon criteria described in the text. MBP, maltose BP; CysBP, cystine BP. See Fig. 2 for other abbreviations.

crisper boundaries for most protein spots, enabling resolution of a number of protein spots that are difficult to see by autoradiography. Despite these differences in methods, most of the proteins on the patterns of Bloch et al. can be seen to correspond to proteins in our patterns.

In Fig. 2, the locations of the periplasmic proteins are indicated in gray on whole cell position patterns for equilibrium (pH 5-7) and also nonequilibrium isoelectric focusing two-dimensional gels. These are patterns for the locations of whole cell proteins on gels, but they do not contain accurate information about protein spot intensities. The nonequilibrium patterns are included to show the more basic proteins that are not seen on the equilibrium patterns. The gray-shaded proteins in Fig. 2 were determined to be periplasmic on the basis of two criteria: 1) release from whole cells by osmotic shock in greater than 50% yield, as judged by comparison between two-dimensional gels of whole cells, shock fluid, and shocked cells (not shown), and 2) release during...
EDTA-lysozyme spheroplasting of whole cells, as judged from
two-dimensional gels of spheroplast supernatant (not shown).
There are a number of other proteins that do not satisfy these
criteria, but that are obtained in significant quantities in
osmotic shock fluid (cf. Fig. 1b) and to some extent in spher-
oplast supernatant. The presence of these proteins appears to
result from small amounts of cell lysis. Addition of Mg$^+$
earlier in the osmotic shock step did in some cases reduce the
amounts of these proteins in shock fluid. On the other hand,
the levels of these proteins in shock fluid also appear to be
somewhat dependent on growth conditions and treatment of
cells before the osmotic shock step.

The equilibrium focusing and nonequilibrium focusing
whole cell position patterns and corresponding locations of
periplasmic proteins are shown in Fig. 3 for wild type K12 and
in Fig. 4 for the W strain HA12. Comparison with the patterns
for strain B and the criterion of greater than 50% release
during osmotic shock were used to determine which proteins
are periplasmic in the patterns for HA12. For K12, we were
unable to get complete release of periplasmic proteins by
osmotic shock. The resulting shock fluid was, however, quite
free of contamination from cell lysis. We therefore
determined the locations of periplasmic proteins for K12 primarily on the
bases of the shock fluid pattern and comparison with patterns
for B and HA12.

The identifications of 10 specific sugar and amino acid
binding proteins are shown on the gel patterns in Figs. 2-4.
The methods used to identify the binding proteins for the
different strains are summarized in Table I. No single strain
had detectable levels of all 10 binding proteins. In Table II,
we have given the x and y coordinates that correspond to the
locations of these binding proteins on the standard gel patterns
published by Bloch et al. (3). These coordinate locations were
obtained by comparison between our patterns and those of

![Fig. 4. Locations of periplasmic proteins on two-dimen-
sional gel position patterns for whole cells of strain HA12
grown on glycerol. Notation is as in Fig. 2. Designations of peri-
plasmic proteins for HA12 are based upon criteria described in the
text. See Figs. 2 and 3 for abbreviations.](image_url)
for induced xylose BP and purified arabinose BP on the denaturing two-dimensional gels (results not shown). We also note that there is only a small amount of carbamylation of other proteins on our normal gel patterns. Arabinose and xylose BP do not appear to be subject to the same regulation. Xylose BP, but not arabinose BP, was induced when strains B and K12 were grown in the presence of xylose. However, we were unable to induce arabinose BP with arabinose in the presence of glycerol for any of the strains studied here.

Ligand binding to renatured binding sites in the two-dimensional gels was used in the identification of a number of proteins. Fig. 5 shows examples of the autoradiographs and protein stain patterns for sections of gels used to renature leucine binding proteins for strains B and HA12. Also shown is an example of a whole gel run under nonreducing conditions. The major leucine binding component is leucine-isoleucine-valine binding protein. To the right of leucine-isoleucine-valine BP in Fig. 5, b and d, is a less prominent leucine binding component. For strain HA12 (Fig. 5, a and b), this protein corresponds to the location of leucine-specific binding protein identified in K12. For strain B (Fig. 5, c and d), the minor leucine binding component has a different charge. We conclude that the PI for leucine-specific BP in strain B is different from that for HA12 and K12. A similar type of charge difference was also encountered for cystine binding protein in strains HA12 and K12, as can be seen in the identification patterns of Figs. 3 and 4. Purified glutamine binding protein comigrated with two periplasmic proteins of different charges in strain HA12. Renaturation experiments demonstrated that both of these proteins bind glutamine (results not shown). The two different charged forms do not appear to be caused by artificial degradation in the two-dimensional gels, since slab isoelectric focusing of purified protein (in the absence of urea) showed the same two spots, and their relative intensities are comparable to the relative intensities of the corresponding proteins in two-dimensional gels of whole cells. The same two periplasmic protein spots appear in strains B and K12 in roughly comparable amounts.

Several of the binding proteins listed in Table I are identified in the gel patterns for one or more strains on the basis of correspondence with the location of the same protein identified on gels of another strain. Proteins identified in this way are listed in parentheses on the gel patterns of Figs. 2–4. Because more than one protein occasionally appears in the same location on gels, it seems wise to treat such correspondences as a tentative guide for the locations of binding proteins until other methods establish the identifications more firmly.

**DISCUSSION**

The classification of periplasmic proteins on whole cell two-dimensional gel patterns for *E. coli* represents the first step in a comprehensive program to classify all whole cell proteins on two-dimensional gels according to subcellular location. Classification of different subcellular fractions serves to extend the general information about physiology and genetic composition that can be obtained from two-dimensional gel electrophoresis of whole cells. Moreover, classification can be used to eliminate some of the ambiguities that can arise in the identification of specific proteins on two-dimensional gel patterns. For example, induction generally causes the elevation of a number of different proteins on two-dimensional gel patterns. It is nevertheless possible to identify many of these proteins if their subcellular classifications are known.

A number of difficulties can be encountered in classifying whole cell proteins on the basis of subcellular location. Distinctions between different subcellular classes are sometimes vague, and some proteins can be located in more than one

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**Fig. 5. Renaturation of leucine binding proteins in equilibrium (pH 5–7) focusing two-dimensional gels of osmotic shock fluid.**

*Fig. 5.* Renaturation of leucine binding proteins in equilibrium focusing two-dimensional gels of osmotic shock fluid for strain HA12: *b,* autoradiogram of *a* before silver staining; *c,* silver stain of gel section used to renature leucine binding sites for strain B; *d,* autoradiogram of *c* before silver staining; *e,* whole gel pattern for osmotic shock fluid from strain B run under nonreducing conditions. *Arrows* on the left in *a–d* point to leucine-isoleucine-valine BP; *arrows* on the right point to leucine BP. Gels contained osmotic shock fluid from 7.6 A600 units of culture grown on glycerol. Electrophoresis and renaturation were performed as described under "Materials and Methods." X-ray film exposures were for 300 h. Patterns in the gel sections are slightly distorted compared to standard whole cell or shock fluid patterns in which lighter protein loads are used and 2-mercaptoethanol is included. The correspondence between these patterns was determined by comparison between complete two-dimensional gels of osmotic shock fluid run under these different conditions (compare *e* with Fig. 1b).

Bloch et al., and were informally confirmed by Prof. Neidhardt's laboratory.

Arabinose binding protein and xylose binding protein do not appear in detectable amounts on gels of cells grown on glycerol. We have used *arrows* to indicate the locations where the principal charged components of these binding proteins would appear on the gel if present. These two proteins are quite similar. They have identical PI values and differ in molecular mass by less than 2000 daltons. Moreover, both proteins frequently appear on gels as a string of three or four charged species, with intensity diminishing in the acid direction. The charge spacing is identical for the two proteins. It appears that this charge heterogeneity develops in *vivo*, since slab isoelectric focusing of the purified arabinose BP (in the absence of urea) shows the same charge heterogeneity as seen

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2 Personal communication by Prof. F. Neidhardt.
class simultaneously or can be located in different classes under different conditions. Furthermore, it is often difficult to establish the validity of operational methods for isolating proteins of any given class. In the work described here, we have attempted to rely on a definition of periplasmic proteins as proteins that are freely soluble in the space between the inner and outer membranes of Gram-negative organisms. We have used osmotic shock and EDTA-lysozyme treatment as methods for releasing periplasmic proteins from cells. Osmotic shock is a convenient method that is frequently used for preparing periplasmic proteins. It generally provides nearly quantitative release of periplasmic proteins. However, it is often accompanied by some cell lysis, and it is a mechanically disruptive method which probably releases some proteins that are more properly considered extrinsic inner or outer membrane proteins. EDTA-lysozyme treatment of cells is a more gentle method for releasing periplasmic proteins from cells, but it often gives poor yields and may cause some structural disruption. In designating the periplasmic proteins on whole cell two-dimensional patterns, we have tried to take a conservative approach. Only those proteins that are released from cells in greater than 50% yield by quantitative osmotic shock or that are released in proportionately high yield by EDTA-lysozyme treatment or mild osmotic shock are designated as periplasmic proteins. We have thus eliminated most proteins that appear in periplasmic proteins by lysis or partial release from the membranes. On the other hand, proteins that are located in both the periplasm and cytoplasm or membranes are probably not identified as periplasmic proteins by these criteria.

For each of the three strains studied here, we find approximately 50 periplasmic proteins out of about 800 total whole cell proteins on the two-dimensional gel patterns. In general there is good agreement between the periplasmic pattern assignments for the different strains. Most of the differences in the patterns result from variations in proteins between strains. Only in a few cases are corresponding proteins designated as periplasmic proteins in one strain but not another. It is tempting to speculate that these few apparent discrepancies may actually reflect architectural differences between strains. In this respect, it is interesting that we were never able to get quantitative release of periplasmic proteins from K12 by osmotic shock, whereas we had no difficulty with the other strains.

There are a number of interesting features in the binding proteins identified here on two-dimensional gels. Arabinose binding protein and xylose binding protein have extremely similar electrophoretic properties (size, pI, and charge heterogeneity). The degree of similarity between these two proteins is much greater than for any other binding proteins identified. On this basis we predict that arabinose and xylose BP have nearly identical sequence homology to each other. Two binding proteins, leucine-specific BP and cystine BP, have different pI values in different strains. There is some possibility that the difference in pI observed for cystine BP in K12 and HA12 is due to mutations that occurred during construction of strain D2W (parent for HA12) from W. These mutations were known to involve a gene that affects some aspect of cystine transport (5). That gene may code for cystine BP. It would therefore be interesting to compare the gel location of cystine BP for strains HA12 and wild type W. The binding of the proteins, xylose BP, arabinose BP, and glutamine BP show more than one charged form on the two-dimensional gels. In the case of xylose BP and arabinose BP, this is clearly the result of protein alteration, as evidenced by the charge profile that consists of several equally spaced species with intensity decreasing in the acid direction. Apparently this alteration occurs in vivo. It is less clear whether protein alteration has occurred for glutamine BP. Only two glutamine binding components are found, and their relative intensities are quite reproducible. Corresponding proteins are seen in strains B and K12. It seems unlikely that there are two genes for glutamine BP, although the possibility cannot be completely discounted.

The procedure that we have developed for renaturation of ligand binding sites in two-dimensional gels offers possibilities for a number of different experiments. It will be interesting to see whether renaturation in denaturing two-dimensional gels works for other types of proteins besides periplasmic binding proteins. Enzymes and membrane receptors are of particular interest, and the procedure could be very valuable for identification of drug and hormone receptors. Histones and several enzymes have already been renatured from one-dimensional sodium dodecyl sulfate gels by other workers (19-25).

Several general comments about the renaturation procedure used here are worthwhile. In order to detect bound ligand above the background ligand concentration in the gel, mass action predicts that the concentration of binding sites in a gel spot must be greater than the effective dissociation constant for ligand. We estimate that the maximum protein concentration in a sodium dodecyl sulfate-gel spot is in the range 1-10 μg. Therefore the effective K_D for ligand must be micromolar or less if binding is to be easily detected in proteins with single binding sites. Periplasmic binding proteins typically have affinities for ligands that require greater than micromolar concentrations of ligand for saturation (26). Once ligand is bound to these proteins, however, the rate of ligand release is quite slow, and so the effective K_D is much less than micromolar. This makes it possible to rinse the gels to remove unbound ligand, thereby reducing background. We have found that renaturation of ligand binding sites in periplasmic binding proteins is ineffective if the proteins have been exposed to sulfhydryl reducing agents. For other proteins, sulfhydryl reduction may not necessarily interfere with renaturation in two-dimensional gels. It is worth noting that several of the proteins renatured from one-dimensional sodium dodecyl sulfate-gels by other workers (19-25) had been treated with sulfhydryl reducers. We are presently pursuing the possibility of renaturing other types of proteins in two-dimensional gels.

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