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QUALITATIVE AND QUANTITATIVE APPLICATIONS OF AFFINITY ELECTROPHORESIS FOR THE STUDY OF PROTEIN—LIGAND INTERACTIONS: A REVIEW

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SUMMARY

A review is given on the present state of the affinity-chromatography-like modification of affinity electrophoresis, i.e. the technique in which a ligand-binding protein is electrophoresed on an affinity gel containing immobilized ligand; mobility on such a gel is decreased as compared to a control, as a result of protein-immobilized ligand interaction. Immobilization of ligands is acheived usually by incorporation of a macromolecular soluble derivative of the ligand (macroligand) into polyacrylamide, agarose or mixed gels. The macroligands can be prepared by substitution of natural or synthetic soluble polymers, by copolymerization of suitable unsaturated ligand derivatives with acrylamide or by substitution of agarose gel beads which can be subsequently dissolved upon heating. The method can be used for detection of ligand-binding proteins, checking the purity and binding homogeneity of purified proteins and for determination of dissociation constants of protein—ligand complexes.

INTRODUCTION

Affinity electrophoresis in a broad sense denotes all techniques in which some kind of biospecific interaction between an electrophoresed component and another component present in the medium (ligand) occurs. This interaction results in a change in electrophoretic mobility of the electrophoresed substance, as compared to its mobility in the absence of the specific ligand in the medium. These methods are used mainly for analytical purposes, i.e. to detect the ligand-binding components present in the sample, to determine the

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binding homogeneity (or heterogeneity) of the ligand-binding component(s), the changes resulting from various treatments and sometimes also for quantitation of the ligand-binding component(s) or for quantitative studies on the protein—ligand complex formation. In the following text we will, for the sake of simplicity, often refer to the protein—ligand interactions, although substances other than proteins can be principally electrophoresed and their interaction with immobilized ligands studied (see Table I).

The ligand may be either soluble or immobilized in the gel medium. If it is soluble, it may form complexes of various properties with the electrophoresed substance. If the ligand is very small as compared to the electrophoresed substance, e.g. a low-mass substrate or inhibitor of an electrophoresed enzyme, the change in the mobility of the enzyme zone will be relatively small or negligible, especially if the ligand has a small or zero charge. Much more marked effects on the electrophoretic mobility are to be expected if the ligand and the ligand-binding protein are of comparable size, if the ligand is highly charged, or if its branched structure enables simultaneous interaction with more than one protein molecule. Then, the interaction may result in a strong decrease or increase in mobility of the electrophoresed substance or even a precipitate may be formed. All these possibilities are employed in some currently existing and fairly widely employed techniques. Most popular are the various modifications of immunoelectrophoresis. In these methods various antigens are electrophoresed, usually in agarose gel media containing incorporated antibodies (i.e. specific immunoglobulins), which especially bind to and usually precipitate with with the antigen. Under suitable conditions, the formation of the zone of antigen—antibody precipitate can be used for a sensitive detection of the antigen in the electrophoresed sample, and even for its quantitation. Usually, the pH of the buffer used and electroendoosmosis of the gel are chosen so that the antibodies have small to zero net mobility. Most popular among these modifications of immunoelectrophoretic techniques are crossed immunoelectrophoresis [1], rocket immunoelectrophoresis [2] and the more recent zone immunoelectroassay [3]. A very similar principle is also the basis of crossed immunoaffinoelectrophoresis and related methods, developed mainly by Bøg-Hansen and co-workers [4–7].

In these methods, glycoproteins are electrophoresed in agarose gel media with incorporated lectins capable of specific complex formation with carbohydrate moieties of the electrophoresed glycoproteins. In some cases, the lectin—glycoprotein interaction results in the formation of a precipitate analogous to the antibody—antigen precipitate [5]: here, the lectin acts as an analogue of an anti-carbohydrate antibody. In other cases, the lectin–glycoprotein complexes remain soluble and the interaction only leads to apparent decrease of the glycoprotein zone mobility. The position of the glycoprotein zone in the lectin-containing gel or control (non-interacting) gel is conveniently detected by electrophoresis of the separated components into the second-dimension gel containing specific precipitating antibodies against the glycoprotein. This is the crossed-immunoaffinoelectrophoresis technique [4], which is widely used both for research on glycoproteins and for clinical diagnostics of various pathological conditions under which abnormal variants of some glycoproteins occur [8]. An interesting extension of this principle is

two-dimensional affinoelectrophoresis: the glycoproteins are electrophoresed in the first-dimension gel containing the first lectin, then in the second (perpendicular) dimension in a gel containing the second lectin, and, finally, the separated glycoprotein spots (or in fact complexes of the lectin with glycoproteins) are blotted onto nitrocellulose membrane impregnated with the specific anti-glycoprotein antibodies [9]. This method has higher resolving power and its principle seems to have wider applicability. Reviews on the immunoelectrophoresis-like variant of affinity electrophoresis were published elsewhere [6, 7, 10, 11].

A similar principle is also the basis of affinophoresis [12, 13]. In this technique a soluble, highly charged macromolecular derivative of a ligand, i.e. affinophore, is incorporated into the gel and its complex with the electrophoresed protein has substantially higher mobility than the uncomplexed protein. This change in the mobility can again be used to detect and to investigate the ligand-binding properties of the electrophoresed component (e.g. an enzyme) and its changes after various treatments potentially affecting the ligand-binding properties.

The last modification of affinity electrophoresis is completely analogous to analytical or quantitative affinity chromatography: the ligand is immobilized within the gel so that the protein (or other substance) electrophoresed is retarded owing to its interaction with the ligand. Various aspects of this technique and its modifications are major subjects of this review. It should be noted that there is no sharp boundary between the immunoelectrophoresis-like and affinity-chromatography-like modifications of affinity electrophoresis, because the immobilization of the ligand in the latter method needs not to be complete when using the macroligand technique of ligand immobilization. The term affinity electrophoresis was created in analogy to affinity chromatography and it was used for the first time by Szylit [14]. Various aspects of affinity electrophoresis were reviewed previously [6, 7, 10, 11, 15–18].

IMMOBILIZATION OF LIGANDS WITHIN THE GEL

Immobilization of ligands for the purposes of affinity electrophoresis can, in some cases, be done by the methods employed currently in affinity chromatography. However, the electrophoretic system has its own special characteristics: usually the separation is performed in a block of gel, which prevents the use of some current methods of covalent coupling of the ligand that are successfully used to prepare small ligand-bearing gel beads for affinity chromatography. It is preferable to use coupling methods and gel media that do not yield high electroendoosmotic flow and do not interfere with subsequent detection of separated components (e.g. protein staining). In most cases, a much lower concentration of immobilized ligand is needed, which is sufficient to cause a retardation of the electrophoresed ligand-binding component than in gels used for preparative affinity chromatography. Methods that can be used for ligand immobilization are given below.

Incorporation of a macromolecular soluble derivative of the ligand (macroligand)

If a sufficiently large macromolecule is added to the solution of monomers

normally used for preparation of polyacrylamide gel, then after the copolymerization reaction a gel is formed in which the macromolecule is entrapped and thus effectively immobilized (Fig. 1a). Clearly, the efficiency of immobilization depends on the ratio of macromolecular size and gel porosity. In many cases natural macroligands exist, e.g. various polysaccharides bearing structures interacting with lectins or some enzymes. Then, the polysaccharide or other macromolecular complex carbohydrate (e.g. blood group substance) is simply incorporated in suitable concentration into the polyacrylamide gel and affinity gel is ready. This approach was taken in early studies employing the affinity electrophoresis technique to identify and to investigate phosphorylases and other polysaccharide-reactive enzymes [19- 22] and in several studies dealing with carbohydrate-specific immunoglobulins [23—26] or with lectins and their derivatives [27—33] (see also Table I).

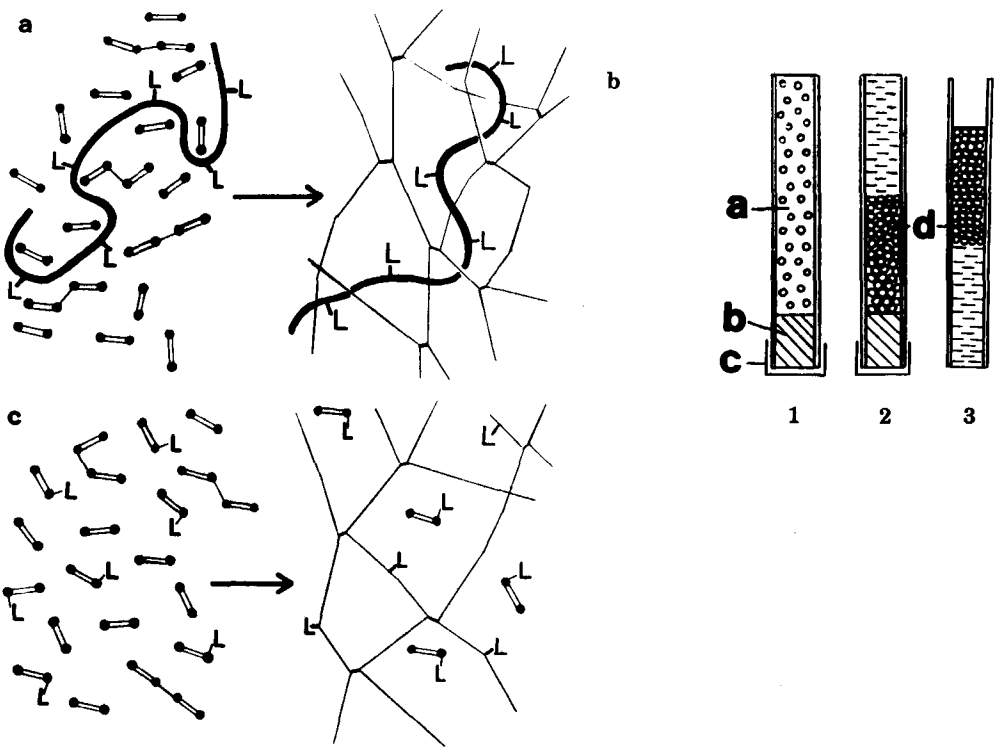

Fig. 1. Schematic representation of techniques used for immobilization of ligands in polyacrylamide gels. (a) The macroligand method: the solution of acrylamide (•=•) and cross-linking agent (•=•—•=•) containing the macroligand (the long structure bearing the ligand residues, L) is polymerized. The macroligand becomes entrapped within the gel matrix (right). (b) Incorporation of ligand-substituted beads into the gel. 1: The suspension of ligand-substituted gel beads in polymerization solution (a) is poured into the glass tubes equipped with a layer of agarose gel (b) supported by a nylon mesh (c). 2: The tube is shortly centrifuged so that a densely packed layer of sedimented beads (d) is formed and the gel is polymerized. 3: The tube is inserted, the agarose layer removed by a stream of water; the affinity gel is ready for electrophoresis. (c) Direct copolymerization of polyacrylamide gel with the copolymerizable derivative of the ligand (•=•^{L}). After copolymerization, the gel matrix contains incorporated ligand molecules, but a portion of the ligand derivative may remain unpolymerized.

Immobilization of any required ligand into the affinity gel by macroligand method requires the ability to couple the ligand to a suitable, soluble, macromolecular carrier. This can be done in several ways. First, a natural macromolecular substance, such as dextran, can be derivatized with the ligand [34—36], as can be linear polyethylene glycols [37]. Coupling of a ligand to such a macromolecule may be complicated by side-reactions leading to cross-linking and thus to insoluble products. Alternatively, synthetic copolymers with suitable reactive groups may be prepared and subsequently derivatized with the ligand [38—40]. Yet another possibility is to prepare the macroligand by copolymerization of acrylamide or some similar monomer with a suitable copolymerizable derivative of the ligand. Derivatives of ligands carrying allyl- or other alkenyl [41—45] or acrylyl [46—48] groups are useful in this respect. Copolymerization of the monomer and ligand derivative in an aqueous solution in the absence of a cross-linking agent yields in a single step a solution of the soluble copolymer containing covalently bound ligand. The ligand concentration can be regulated by the concentration of the monomeric derivative in the reaction mixture and the length of copolymer chain by polymerization initiator concentration. After the copolymerization reaction, non-reacted monomers or low-molecular oligomers are removed by dialysis; the copolymer (macroligand) can be lyophilized and stored for future use. This method is simple and convenient but preparation of a suitable polymerizable ligand derivative may not always be simple (though it is very simple, e.g., in the case of allyl α-glycosides). This method was used to prepare affinity gels containing sugars [41, 42, 45], hydrophobic ligands [46], nucleotide derivatives [43, 44], tertiary amine derivatives [47, 48] or dyes reactive with specific nucleotide sequences [37].

Incorporation of ligand-substituted gel beads

Instead of soluble macromolecular ligand carriers, still larger particles, i.e. gel beads carrying the ligand residues, may be incorporated into the gel matrix. Essentially, the same types of ligand-substituted gel beads can be used as those employed in affinity chromatography, assuming that these gel beads do not inhibit formation of the embedding (polyacrylamide) gel matrix and that they do not interfere severely with subsequent steps, e.g. owing to high electroendoosmosis or staining of separated components. An advantage of this method is that numerous existing simple and rapid methods for ligand binding to the gel beads can be used. However, some methods, e.g. the cyanogen bromide activation method, may yield derivatives of limited use because of their high electroendoosmosis or strong staining with common protein stains. It seems that agarose or dextran gel beads activated by periodate oxidation and subsequently coupled with the amino-ligand by reductive amination are most suitable [49, 50]. Also it is necessary to prevent uneven sedimentation of the gel beads in the polymerization solution before the gel matrix is formed. This can be achieved using a simple trick [49] leading to rapid formation of a uniform densely packed layer of gel beads at the bottom of the gel rod, which becomes the top of the affinity gel in the final arrangement (Fig. 1b).

Meltable derivatives of agarose gel

This method is again a variant of the macroligand technique; here, the

soluble macromolecular carrier of a ligand is the polysaccharide agarose, which is soluble at elevated temperatures. A great advantage of this ligand carrier is that commercially available agarose gel beads can be easily coupled with a ligand (purification of the macroligand is in this case achieved by simple washing of the derivatized gel beads), then solubilized by heating them on a hot water bath and mixing at suitable concentration with prewarmed solutions of underivatized agarose solution. After pouring the solution into the tubes (or flat-bed casettes), the affinity gel is formed after cooling. This method yields agarose-based affinity gels in which the macroligand is efficiently immobilized apparently by incorporation of the ligand-substituted agarose chains into the bundles of unsubstituted agarose chains; it should be noted that incorporation of other macroligands (dextran, synthetic macromolecules) into agarose-based affinity gel is otherwise expected to be difficult due to the great porosity of agarose gels. Preparation of agarose-based affinity gels in this way is extremely simple. However, it must be borne in mind that many modification reactions of agarose gel yield a cross-linked product, which cannot be melted upon further heating. This is especially true for cyanogen bromide-activated gels. A convenient method of preparation of meltable agarose bead derivatives is activation by periodate oxidation followed by reductive amination coupling of an amino ligand [49], which yields derivatives completely soluble upon heating. Similarly, meltable derivatives can be prepared by coupling reactive triazine dyes directly with underivatized agarose gel beads [51].

Meltable agarose gel derivatives can be used also for immobilization of ligands in polyacrylamide gels [49]. In this case, the warm solution of ligand-substituted agarose is added to a prewarmed solution of acrylamide–bisacrylamide monomers as normally used for preparation of polyacrylamide gels, and the gels are left to polymerize at elevated temperatures and then cooled down (the polyacrylamide gel should be formed before the agarose gel is allowed to form). In this technique, which is in fact the commonly used technique of composite acrylamide–agarose gels, lower concentrations of polymerization catalyst must be used (due to the elevated temperature used), and the gels must be suitably thermostated during polymerization. An optimal concentration of polymerization catalyst must be found empirically in each particular case, because some ligands may inhibit polymerization and thus counteract the effect of elevated temperature.

Direct incorporation of a polymerizable ligand derivative into polyacrylamide gel

In this method, a polymerizable derivative of the ligand is added in suitable concentration to the solution of acrylamide–bisacrylamide monomers, and the gels for electrophoresis are prepared in the usual way. The ligand becomes incorporated directly into the gel matrix (Fig. 1c). Here, the immobilization is covalent and ligand molecules are distributed quite randomly within the gel, which are the features distinguishing this method positively from the macroligand method in which imperfect immobilization may occur in highly porous gels and where islets of ligands (attached to one macroligand molecule) occur. This method has been tested so far only with allyl glycosides as the

polymerizable ligand derivatives [52, 53], and here a serious problem was that copolymerization of these allyl derivatives with acrylamide monomers was incomplete; large amounts of unpolymerized allyl glycoside remained in the gel and had to be removed by washing. Then, the gel rod had to be inserted back into the glass tube in which electrophoresis was performed. For this reason, the method was abandoned after the introduction of synthetic macroligands. However, it seems very likely that suitable acrylyl or similar derivatives would copolymerize quantitatively with acrylamide and then this technique might become the method of choice for preparation of affinity gels. It should be noted that a similar approach, i.e. quantitative copolymerization of acrylyl derivatives of various ampholytes with acrylamide—bisacrylamide monomers, is successfully employed for the preparation of gels with immobilized pH gradients [54].

CONDITIONS OF ELECTROPHORESIS

The conditions used for affinity electrophoresis are basically the same as those used in normal electrophoretic methods. The amount of immobilized ligand in the gel is usually low, so that in most cases it does not markedly affect the properties of the gel. Nevertheless, it should be stressed that control (non-interacting) gels, which are always run in parallel with the affinity gels, should be as similar to control gels as possible. It is desirable to use control gels containing the same amount of immobilized macroligand (i.e. a molecule very similar to the ligand immobilized in the affinity gels, but of no affinity to the protein) as the comparable affinity gel. This is in order to eliminate the effects of the immobilized ligand or its carrier on general properties of the gels, such as porosity, electroendoosmosis, etc. Some ligands may, for example, to some extent inhibit polyacrylamide polymerization and thus the properties of gels may be changed. These non-specific effects of affinity gels should be checked in advance.

There are several other points which should be considered. First, buffers or other components of the electrophoretic system that might interfere with the protein—ligand interaction must be avoided (e.g. concentrated urea, sodium dodecyl sulphate, etc.); borate buffers usually cannot be used if an interaction with immobilized carbohydrate is to be studied, similarly sucrose must be omitted from the sample solution or stacking gel in the same situations. Second, some kinds of affinity gels may be strongly stained with common protein stains; then alternative methods of localization of separated components may be necessary, such as autoradiography, specific enzyme staining or perhaps a blotting procedure.

Affinity electrophoresis may be performed in slabs or rods of the gel. The slab gel arrangement is useful if several samples are to be compared side by side on a single gel. If one sample is to be tested on several affinity gels (differing in the nature and/or concentrations of the ligands), rod gels are recommended. The rod gels are also more economic, which may be important in the case of expensive ligands.

The porosity of the affinity gel does not seem to be very important, at least if the technique is to be used for simple qualitative purposes; large-pore gels are preferable in quantitative studies on protein—ligand interactions, when

undesirable non-specific effects of the gel matrix should be minimized. In other cases, gel porosity should be optimal for resolution of various components of the sample. It is interesting that the apparent strength of the protein—ligand interaction may increase with increasing gel concentration [55].

Both continuous and discontinuous buffer systems can be used; the former are preferable in quantitative studies on protein—ligand interactions. Discontinuous buffer systems in which concentration of the protein zones occurs at the beginning of the separation give sharper resolution of narrow protein zones but the concentration of protein in these zones may be too high, which is at variance with one of the basic principles of affinity electrophoresis (see below), especially if it should be used for quantitative purposes.

The principle of affinity electrophoresis can be used also in combination with isoelectric focusing (IEF) [56]. Any of the previously discussed methods of ligand immobilization may be used, but the IEF system is much more sensitive to increased electroendoosmosis in affinity gels. Therefore, high concentrations of immobilized charged molecules must be avoided. IEF has a very high resolving power, which can be easily modified by the pH range used. This may be especially useful if ligand-binding components of complex mixtures are to be identified. Affinity IEF may be advantageous in many cases of such qualitative applications (see below), as most protein samples can be focused with high resolution, whereas looking for a suitable electrophoretic system may be a time-consuming trial and error process. However, affinity IEF can of course be used only for qualitative purposes, not for estimation of dissociation constants of protein—ligand complexes. Optimal results (i.e. maximal retardation of the ligand-binding protein as compared to a control, non-interacting gel) can be expected if relatively low voltage and short focusing times are used; high voltage and prolonged focusing may apparently overcome the protein—ligand interaction, so that the ligand-binding protein will finally focus near its correct p*I* observed on the control gel [56] (Fig. 2a).

Also, the feasibility of affinity isotachophoresis has been demonstrated

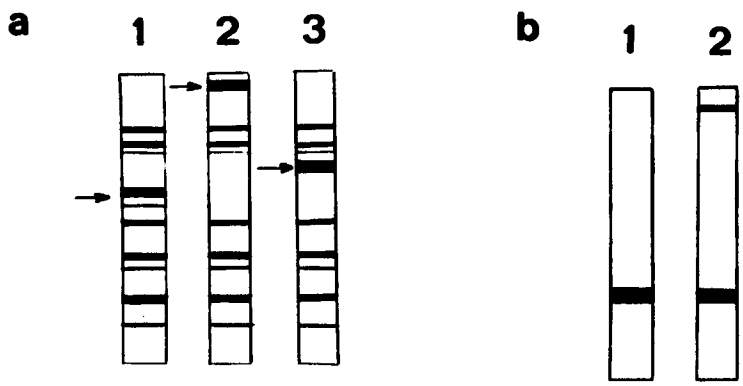

Fig. 2. Schematic representation of the results of affinity isoelectric focusing (a) and affinity isotachophoresis (b). Arrows indicate the positions of ligand-binding protein bands. Samples were applied at the top of the gels. (a) 1 = Control gel; 2 = affinity gel after relatively short time of focusing; 3 = affinity gel after prolonged focusing or focusing at high voltage. (b) 1 = Control gel (all proteins migrate in a poorly resolved stack zone); 2 = affinity gel (ligand-binding components are extracted from the stack and are strongly retarded).

[57]. Here, the conditions can be found such that non-interacting proteins will migrate, unseparated, rapidly with the front, while the ligand-binding proteins will be strongly retarded due to their interaction with the immobilized ligand (Fig. 2b). The advantage of this method is that it can detect very clearly even small amounts of the ligand-binding protein in a very complex mixture. It is not clear how general this method is; it seems that in some cases the high-voltage gradient in the stack zone is capable of overcoming any retardation of the ligand-binding protein caused by the protein—ligand interaction. This aspect would require further study.

QUALITATIVE AND QUANTITATIVE APPLICATIONS OF AFFINITY ELECTROPHORESIS

The affinity-chromatography-like modification of affinity electrophoresis discussed here can be used mainly for detection and identification of ligand-binding component(s) present in the sample. To this aim, the pattern obtained on a suitable control (non-interacting) gel and specific-affinity gel is compared; specific interaction is manifested by retardation of the ligand-binding component on the affinity gel as compared to the control gel. This may be useful in several situations. In short, it can be used: (1) for demonstrating the presence and the number of ligand-binding components in a complex mixture [19–21, 26, 35, 47, 48, 56]; a very efficient method may be the two-dimensional affinity electrophoresis described by Takeo and co-workers [58, 59], in which the sample is first separated by IEF and then the IEF gel is used as a sample for second-dimension electrophoresis on a slab of affinity gel; (2) for checking the binding homogeneity of purified preparations and to detect inactive admixtures [56, 60]; (3) for checking the course and results of modification reactions potentially affecting the ligand-binding site [30, 31, 60–63]; (4) for evaluation of the effects of various factors, such as pH [45, 64–66], ionic strength [67], temperature [29, 40], detergents [68] or spacer length [38, 39] on the ligand-binding activity of the protein; (5) for pre-testing materials intended for use in preparative affinity chromatography [4, 69].

In some of these applications, at least a semiquantitative evaluation of the results is possible; it can be shown clearly which component interacts more strongly or under which conditions the interaction is stronger or weaker compared to standard conditions. In fact, quite early simple relationships have been introduced that are suitable for quantitative evaluation of the strength of protein—immobilized ligand interactions in terms of dissociation constants [21, 22, 42]. Although different notations have been used by different authors, the basic equation used in this respect can be written as:

$$\frac{d}{d_0 - d} = \frac{K_i}{c_i} \tag{1}$$

or, in equivalent form

$$\frac{1}{d_0 - d} = \frac{K_i}{d_0 c_i} + \frac{1}{d_0}$$

where d and d_0 are the mobilities of the protein band on the affinity or control gel, respectively; K_i is the dissociation constant of the protein—immobilized ligand complex; and c_i is the concentration of the immobilized ligand (see also Fig. 3). Thus, K_i is obtained as the slope of the $d/(d_0 - d)$ vs. $1/c_i$ plot, or as the intercept of the $1/(d_0 - d)$ vs. $1/c_i$ linear plot with the abscissa axis (Fig. 3).

In addition to the possibility of studying the interaction of the electrophoresed protein with an immobilized ligand, it is also possible to incorporate into the gel (in addition to immobilized ligand) a ligand in a free, soluble form.

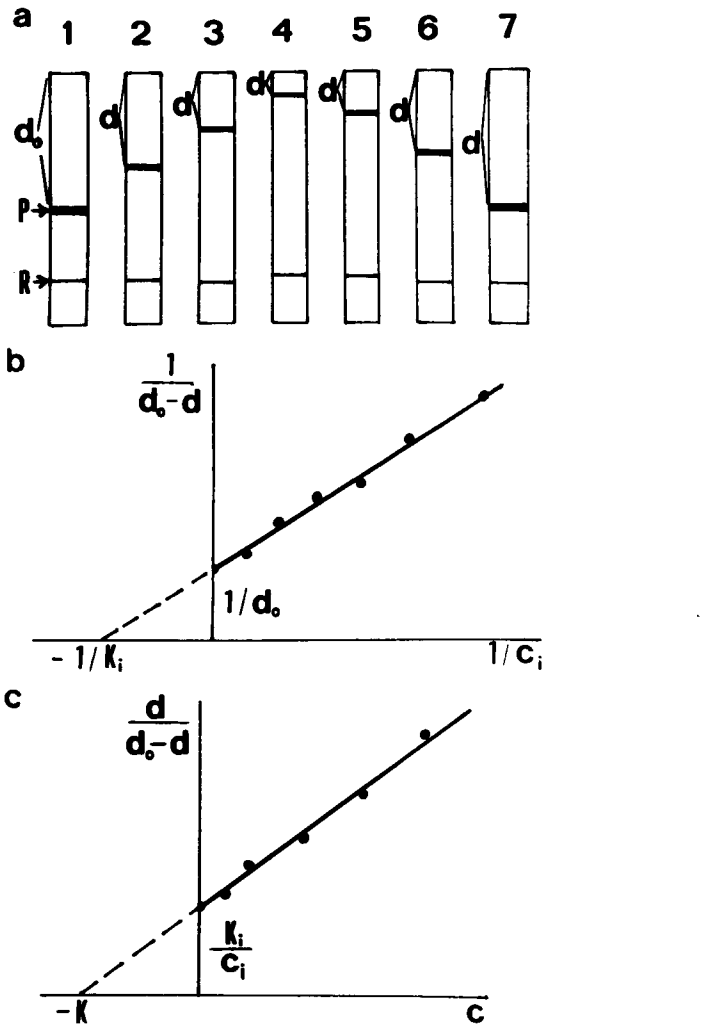

Fig. 3. Quantitative applications of affinity electrophoresis. (a) Schematic representation of a series of affinity gels for evaluation of dissociation constants. 1 = Control gel; 2—4 = affinity gels containing increasing concentrations of the immobilized ligand (c_i); 5—7 = affinity gels; c_i the same as in gel 4 but with increasing concentration of soluble ligand (c). R = a reference high-mobility substance non-interacting with the affinity gel, which serves as an internal mobility standard to which the mobilities d_0 and d of the ligand-binding protein (P) are normalized in order to eliminate a slight variation among individual gels. (b) Graphical estimation of K_i according to eqn. 1. (c) Graphical estimation of K according to eqn. 2.

This soluble ligand will compete with the immobilized ligand for the electrophoresed protein, which will result in a change in migration velocity. Again, the dependence of mobility of the protein on concentration of soluble ligand (at constant concentration of immobilized ligand) can yield quantitative information on the strength of the protein–soluble ligand interaction [42]. This method works best with soluble neutral (uncharged) ligands like sugars, but under suitable experimental conditions (e.g. an arrangement similar to that used in affinophoresis [12, 13], charged soluble ligands could also be used [43,44]. Note that the charge of the immobilized ligand is not very important. Under several simplifying conditions, discussed in more detail in the following paragraphs, eqn. 2 or its equivalent forms can be used for evaluation of the dissociation constants of the protein–soluble ligand complex:

$$\frac{d}{d_0 - d} = \frac{K_i}{c_i}\left(1 + \frac{c}{K}\right) \tag{2}$$

(the meaning of symbols used is the same as in eqn. 1, c is the soluble ligand concentration). Thus, K is obtained as the intercept of the linear plot $d/(d_0 - d)$ vs. c with the abscissa axis (Fig. 3).

Although the relationships (eqns. 1 and 2) are very simplified and neglect many potentially complicating factors, the dissociation constants obtained on their basis are very useful, at least for comparative purposes: the ratio of K values for different proteins (e.g. isoenzymes or isolectins) can be reliably estimated or the change in the K value upon some chemical treatment of the protein or as a result of changed conditions (pH, ionic strength, temperature, etc.) can be estimated. The relationship of these values to true thermodynamic intrinsic dissociation constants may not be quite certain in some cases: better understanding of this relationship is dependent on a more rigorous theoretical analysis of the system accounting for potentially complicating factors, as shown in the following paragraphs. In spite of these reservations, affinity electrophoresis appears to have several advantageous features both for qualitative and quantitative (or at least semiquantitative) applications. (1) Simplicity and economy: no special instrumentation in addition to the gel electrophoresis equipment is needed and many samples can be run and evaluated simultaneously; these samples can be of various degrees of purity, even very crude preparations can be used to obtain quantitative information on protein–ligand interaction. (2) Several different ligand-binding components present in the sample can be studied simultaneously on a single gel. (3) Even very weak interactions can be (semi)quantitatively studied, because very high immobilized ligand concentration can easily be achieved, as well as interactions of medium strength. It seems that difficulties may be encountered in studies of extremely strong interactions (see below).

The major practical or technical limitations of this method are: (1) the necessity to immobilize the ligand within the gel; (2) the necessity to choose a suitable buffer system in which the mobility of the component to be studied is sufficiently high, so that the difference between the control and affinity gel is clearly discernible (therefore, it is not possible to work too close to the pI; the use of affinity IEF might sometimes be the simplest solution if qualitative

TABLE I

SYSTEMS STUDIED BY AFFINITY ELECTROPHORESIS

Affinophoresis [12, 13] and immunoelectrophoresis-like affinity electrophoresis (see Introduction) not included in the table.

Electrophoresed component	Immobilized ligand	Free (soluble) ligand	References
Amylases, phosphorylases	Starch, glycogen		19—22, 65, 70—79
		Oligosaccharides	80
Dehydrogenases, kinases, serum albumin	Cibacron Blue		35, 51, 56, 81, 82
Dehydrogenases	2′-AMP		43
Lactate dehydrogenase	5′-AMP	Nucleotides	39, 44
Trypsin	*p*-Aminobenzamidin		38, 49, 50
Cholinesterase	Procaine, tertiary amine derivatives		47, 48, 55, 62
Ribonuclease	UDP		49
Immunoglobulins	Dextrans	Oligosaccharides	23, 24, 26
	Fructan	Oligosaccharides	25
	DNP, TNP groups		58, 40
	D-GalNAc	D-GalNAc	83
α-D-Galactosidase	D-Gal	Sugars	84
Galactose oxidase	D-Gal	Sugars	61
Lectins	Blood group substances	Sugars	27, 28, 30—32
	Glycoproteins		85, 86
	Polysaccharides	Sugars	29, 32, 66, 87
	Various sugars	Sugars	34, 42, 45, 49, 50, 52, 53, 56, 57, 60, 63, 64, 67, 68, 88—97
Insulin	Sugars		98
Glycoproteins	Lectins		99—101
Albumin	Antibodies		102—104
Various proteins and enzymes	Hydrophobic groups		36, 46, 69, 105
DNA	Dyes		37, 49
	Agarose		106

information is sufficient); (3) exact interpretation of the values of dissociation constants obtained by this method may sometimes be complicated, as discussed in more detail below. Nevertheless, these values can certainly be safely used for quantitative comparative purposes.

The list of systems for which affinity electrophoresis (the affinity-chromatography-like modification) has been used is given in Table I.

THEORETICAL ASPECTS: LIMITATIONS OF THE QUANTITATIVE APPLICATIONS

Closer inspection of the factors involved in the affinity electrophoresis system indicates that simple eqns. 1 and 2 are exactly valid only under several assumptions: (a) immobilization of the ligand is complete (the protein–immobilized ligand complex has zero mobility); (b) mobility of the protein—soluble ligand

complex is identical to that of free uncomplexed protein; (c) concentration of the protein in the migrating zone is much lower than the concentration of the free and immobilized ligand; (d) complex formation and dissociation are very rapid reactions; (e) the protein molecule contains a single ligand-binding site; (f) all molecules of the immobilized ligand are equally accessible to interaction with protein, i.e. effective concentration of the ligand is identical to its total concentration; (g) microdistribution of the immobilized ligand is homogeneous (statistical); (h) the influence of electric field on K and K_i is negligible; (i) the influence of the gel nature on K and K_i is negligible.

At present, basic theory of affinity electrophoresis exists [88, 107, 108], which enables the evaluation of the effects of violation of these assumptions on the results of affinity electrophoresis. Experimentally, the most important conclusions of this theoretical analysis are given below.

(a) Dissociation constants K_i and K can be estimated even if immobilization of the ligand is not complete and if the mobility of the protein–mobile (soluble) ligand complex differs from that of the free protein. This is especially important in the immunoelectrophoresis-like modification of affinity electrophoresis in which a lectin is freely incorporated but not covalently immobilized into an agarose gel and also if the macroligand is not sufficiently large to ensure complete immobilization by physical entrappment within the polyacrylamide gel matrix. In this case, the apparent dissociation constant K_i is determined according to eqn. 3 [107, 109]:

$$\frac{1}{d_0 - d} = \frac{K_i}{d_0 - D_i} \cdot \frac{1}{c_i} + \frac{1}{d_0 - D_i} \tag{3}$$

where d_0 and d are the mobilities of the electrophoresed protein on the control and affinity gel (containing partially immobilized ligand in concentration c_i), respectively, and D_i is the mobility of the protein–macroligand complex.

The general case, i.e. affinity electrophoresis in a gel containing a semi-immobilized ligand (concentration c_i, mobility of its complex with the protein being D_i) and a mobile ligand (concentration c, mobility of its complex with protein being D), is described by eqn. 4, which includes eqns. 1–3 as special cases [107]

$$\frac{d}{D - d} = \frac{K_i D}{K(DK_i + Dc_i - K_i d_0 - c_i D_i)} c + \frac{K_i d_0 + c_i D_i}{DK_i + Dc_i - K_i d_0 - c_i D_i} \tag{4}$$

(b) The plot $1/(d_0 - d)$ vs. $1/c_i$ (or $d/(d_0 - d)$ vs. $1/c$) is linear (eqn. 5), even in the case of high protein concentrations (similar to c_i or even higher), if this protein concentration is constant during the experiment. This can best be achieved by using a large volume of the sample (protein concentration A), i.e. under conditions analogous to frontal affinity chromatography [110], and measurement of the protein zone front position as d (or d_0) [90].

$$\frac{d}{d_0 - d} = (A + K)\frac{1}{c_i} \quad \text{or} \quad \frac{1}{d_0 - d} = \frac{A + K_i}{d_0} \cdot \frac{1}{c_i} + \frac{1}{d_0} \tag{5}$$

Thus, K_i can be estimated even at high protein concentrations, as the value of A is known. It should be noted that, in the frontal arrangement, the value of

($A + K_i$) is obtained instead of K_i using conventional plotting according to eqn. 1; this value will be close to K_i at negligible protein concentrations. In the normally used zonal arrangement, instead of the frontal one, diffusion, stacking and zone sharpening owing to protein–ligand interaction will affect the protein concentration in a complex way, so that deviations from eqn. 5 are expected which may produce errors in K_i estimation under conditions of non-negligible protein concentration.

(c) Knowledge of the kinetics of protein–immobilized ligand interaction is essential for the applicability of affinity electrophoresis [108]. Affinity electrophoresis, as well as quantitative chromatography and similar methods, is a dynamic, not an equilibrium method, in which many consecutive near-equilibria are established during the run. Only if the kinetics of the complex formation/dissociation is sufficiently rapid, can the state very close to equilibrium be established in every elementary step. If the rate of complex dissociation is small, i.e. the lifetime of the complex is long, the departures from the near-equilibrium behaviour will cause broadening and asymmetry of the profile of the ligand-binding protein zone, as predicted by the theory [108]. If the half-life of the complex $\tau_{1/2}$ is much smaller than the entire time of electrophoresis T (at least 100–1000 times), the kinetic effects can be neglected; if $T/\tau_{1/2}$ is only 10—100 times smaller, a considerable broadening of the zone is to be expected and the half-width $\delta_{1/2}$ of the (essentially Gaussian) zone could be principally used for measurement of rate constants k_1 and k_2 of complex formation and dissociation reactions [108]. For even more long-lived complexes, broadening and smearing of the zone would prevent evaluation of the degree of retardation and K_i estimation, and for kinetically stable complexes electrophoretic separation of bound and free protein after equilibrium establishment is possible. Obviously, under the conditions normally used ($T \approx$ h), most protein–ligand complex dissociation reactions can be considered to be very fast ($\tau_{1/2}$ usually < 1 s); only for very stable complexes, e.g. some strong antibody–antigen complexes, may $\tau_{1/2}$ be of the order of 10^3 s, which may impose limitations on the use of affinity electrophoresis in such cases.

(d) If kinetic effects are negligible, a sharpening of the interacting protein band on the affinity gel is predicted (and is commonly observed) as compared to the control gel, which is simply due to the fact that the protein is in a non-diffusible state for a fraction of time [88].

(e) The effects of multiple (identical, independent) ligand-binding sites in the protein molecule depend substantially on the concentration and distribution of immobilized ligand molecules in the affinity gel, i.e. on the possibility of simultaneous multiple interaction of the protein with two or more immobilized ligand molecules. If the mean distance between two neighbouring immobilized ligand molecules is considerably greater than the dimensions of the protein molecule, simultaneous interaction with two or more immobilized ligand molecules is very unlikely. In this case, eqns. 6 and 7 can be used for evaluation of dissociation constants which are analogous to eqns. 1 and 2, except that K_i/n or K/n are obtained instead of K_i or K ($n = 1$ for a monovalent protein):

$$\frac{1}{d_0 - d} = \frac{K_i}{n d_0 c_i} + \frac{1}{d_0} \qquad (6)$$

$$\frac{d}{d_0 - d} = \frac{K_i}{nc_i}\left(1 + \frac{c}{K}\right) \tag{7}$$

Thus, under these conditions, an n meric form of a monovalent protein will interact apparently n times more strongly with the affinity gel than the monovalent form, whereas apparently identical strength of interaction with free (mobile) ligand (in terms of K) will be found. If simultaneous interactions with two or more immobilized-ligand molecules do occur (either due to higher density of the immobilized ligands or large dimensions of the protein molecule), the plots $1/d_0 - d)$ vs. $1/c_i$ and $d/(d_0 - d)$ vs. c will be curvilinear and estimation of K_i (K_i/n) or K will be difficult. Semiquantitative analysis of probability of simultaneous multiple interactions for various proteins on affinity gels indicates that these complicating multiple interactions may be negligible in many cases [88]. Monovalent proteins are in this respect ideally suited for this method. The behaviour of a more complex system involving multivalent protein with non-equivalent or interacting ligand-binding sites has not been theoretically analysed as yet.

(f) Due to steric hindrance and inaccessibility of some immobilized ligand molecules, the effective concentration of immobilized ligand c_i necessary for estimation of K_i is generally lower than the total analytical concentration. The value of effective c_i can be estimated from the dependence of protein mobility on its concentration at fixed c_i, again in the frontal arrangement [88, 90].

$$\frac{d}{d_0 - d} = \frac{1}{c_i} \cdot A + \frac{K_i}{c_i} \quad \text{or} \quad \frac{1}{d_0 - d}\,\frac{1}{c_i d_0} \cdot A + \frac{K_i + c_i}{c_i d_0} \tag{8}$$

(A is the concentration of protein in the sample before entering the affinity gel; eqn. 8 is identical to eqn. 5, but A instead of c_i is the variable.)

(g) The effects of non-homogeneous distribution of the immobilized ligand (e.g. an islet-like distribution in the case of affinity gels prepared from the macromolecular carriers of the ligands) cannot be evaluated exactly at present; they should be considered especially in the case of multivalent proteins [88].

(h) Little is known about the possible effects of electric field on the values of dissociation constants K_i and K. There is some evidence to suggest that a high-potential gradient may strongly contribute to dissociation of the protein–immobilized ligand complex [111] that is practically employed in preparative electrophoretic desorption from affinity carriers [112–114].

(i) The concentration of the gel matrix (agarose and especially polyacrylamide) in real gels is far from negligible. The structure of the gel imposes limitations on the free diffusability of the electrophoresed protein and it may also restrict the accessibility of some immobilized ligand molecules. Experimental evidence surprisingly indicates that the apparent strength of the protein interaction with immobilized ligand increases with increasing gel concentration [55], which may be explained by stabilization of complexes due to restricted diffusion of the protein and by forced closer contact between the protein and immobilized ligand.

In conclusion, the dissociation constants obtained by affinity electrophoresis (especially K_i) should be considered apparent constants in the sense that they

may differ slightly from true thermodynamic constants. This departure from true K or K_i is due to the effects of the above-mentioned factors, which may be difficult to control. However, these constants are intrinsic constants, as they concern the interaction with individual ligand-binding sites. In spite of these reservations, dissociation constants of complexes mainly with soluble ligands, as estimated by affinity electrophoresis, are in relatively good agreement with those determined by other methods, which indicates that the systematic errors owing to violation of simplifying assumptions may not be serious. Nevertheless, the limitations discussed here should always be taken into consideration, and the meaning of absolute values of K_i and K should be interpreted cautiously. It is safest to use the quantitative results of affinity electrophoresis for comparative purposes: to compare the strength of interaction of a protein with different ligands or to compare the strength of interaction with a single ligand under different conditions. It should be noted that most other methods used for the evaluation of dissociation constants of protein—ligand complexes (especially dynamic methods) also have limitations of their own, which are sometimes tacitly neglected.

CONCLUSIONS AND PROSPECTS

It can be concluded that both practical and theoretical bases of the method are established. Reliable methods of ligand immobilization have been marked out, various modifications have been employed in different protein—ligand systems, and potential application of limitations of the method are known. Complementary methods, i.e. the immunoelectrophoresis-like modifications of affinity electrophoresis [4—7, 10, 11] and affinophoresis [12, 13], which were not treated in this review, enable the study of some intermolecular interactions that cannot be studied properly by the affinity-chromatography-like method.

We feel that the major field of future development of this method is finding ways of using it for studies on macromolecule—macromolecule interactions, e.g. protein—protein or protein—nucleic acid interactions. Although this type of interaction can be properly studied in special cases by the immunoelectrophoresis-like modification, it would certainly be better to have simple methods of immobilization of one macromolecular partner on a suitable carrier. Presently existing methods, i.e. immobilization of macromolecules on gel beads and their incorporation into the gel block as used in several studies so far [69, 85, 86, 99—101, 105], do not seem to be generally applicable, especially for substances available in minute amounts. Our preliminary experiments indicate that nitrocellulose membrane or similar materials might be suitable for this purpose. A potentially great advantage of the use of affinity electrophoresis for the study of macromolecule—macromolecule interactions should be the ability to detect even weak interactions, which are difficult to detect using most current equilibrium-binding tests. It would also be very important to explain quantitatively the effects of the electric field on intermolecular interactions with respect to their influence on the values of dissociation constants as determined by affinity electrophoresis and with respect to the use of preparative electrophoretic desorption.

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