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Review

Utilization of enzyme–substrate interactions in analytical chemistry

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Abstract

Enzymes are capable of a highly specific interaction with a variety of substances including their respective substrates. This review summarizes how such interactions may be used in analytical (bio-)chemistry, e.g., for the elucidation of the binding mechanism, the determination of the binding strength, the carting of the binding site, or the screening of possible substrate/inhibitor molecules. Possible assay formats such as analytical affinity chromatography, affinity capillary electrophoresis (ACE), conventional affinity gel electrophoresis (AEP), and related techniques are discussed together with examples of recent applications. In addition a brief section on enzyme–substrate reactions as tools in analytical chemistry is included, since these are perhaps even more important to analytical (bio-)chemistry. The development and application of bioanalytical systems and especially biosensors in various fields including medicine, biotechnology, agriculture, defense and foodstuffs are considered. © 1999 Elsevier Science B.V. All rights reserved.

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Contents

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1. Introduction

Enzymes are biocatalysts. Their metabolic function is to exert a specific influence on a certain biochemical interaction usually in a very complex environment, for example inside a cell or microorganism. Evolution has thus produced catalysts of a specificity and efficacy that is at present unthinkable for even the most advanced man-made catalysts. Multi-enzyme complexes or cascades, Ping-Pong mechanisms, and other connected systems are used to push and pull certain multi-step reactions towards the desired end. Mass transfer phenomena are used to fine-tune the result.

The specificity of the enzymatic reaction is usually imagined as being the result of a steric fit. The ''key and lock'' or the ''induced fit'' image are evoked to illustrate the underlying principle. Various types of weak and usually non-covalent interactions can be formed between biologicals. Among them hydrogen bridges, a number of van der Waals type and electrostatic interactions are the most prominent. Only when several of these interactions can occur simultaneously because the putative interaction points between the two molecules coincide in a three-dimensional framework, ''binding'' takes place, Fig. 1. The strength of the resulting bound can cover several orders of magnitude and in some cases, for example in certain hormone–receptor pairs, approach that of a covalent one.

Incidentally, even though most biochemistry books tend to depict the enzyme–substrate reaction as that of a large molecule (the enzyme) interacting with a small one (the substrate), not all enzyme substrates are small. In fact an enzyme such as a restriction endonuclease may appear tiny in comparison to its substrate the DNA molecule. The same can be said for some polysaccharide degrading enzymes.

The analytical questions and issues connected to enzyme interactions with substrates, inhibitors, and co-enzymes are manifold. One aspect is the enzyme as subject of analysis. This may concern the elucidation of the binding mechanism, the determination of the binding strength, the carting of the binding site, or the screening of possible substrate/inhibitor molecules. The latter aspect is of especially high interest to the pharmaceutical industry. Since many metabol-

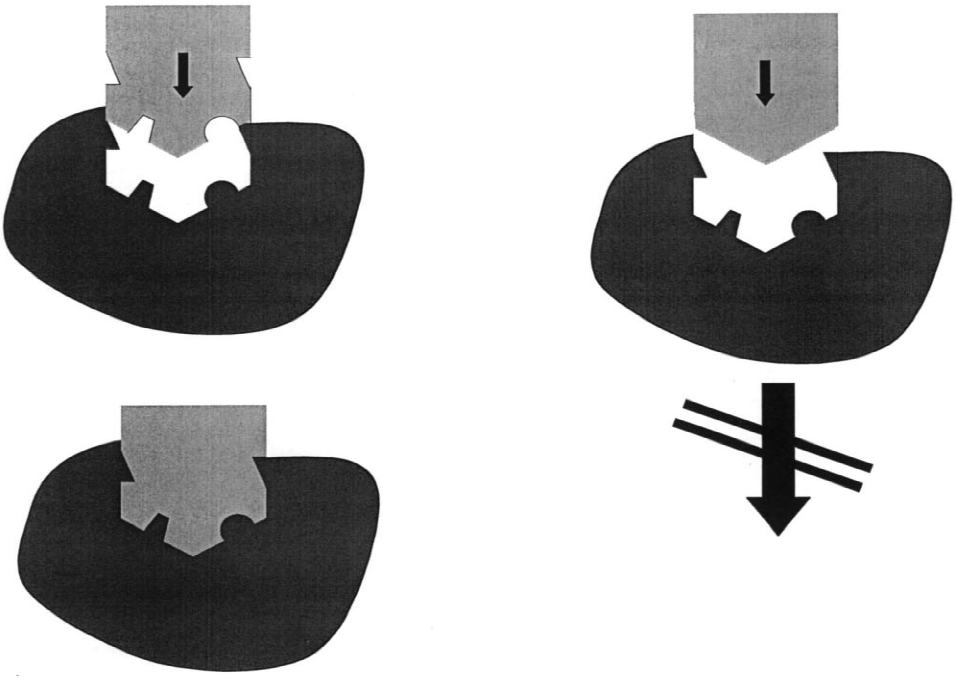


Fig. 1. Illustration of the interaction between an enzyme and its substrate.

ic functions and reactions are controlled enzymatically, the classical pharmaceutical drug is a small molecule capable of interacting with an enzyme. Many of the recently published papers on the analysis of enzyme–substrate interactions thus deal with drug screening or the investigation of certain reactions within metabolic pathways. However, since the topic of drug–protein interaction has only recently been the topic of several excellent reviews including the one in 1997 by Hage and Tweed in the *Journal of Chromatography B* [1], this interesting subject will not be explicitly discussed here.

Enzyme–substrate reactions as tools in analytical chemistry are perhaps even more important to analytical biochemistry. For a long time ''enzyme'' was almost a synonym for the biological part of any biosensor and to this day the selectivity of enzymes for their substrate together with the enormous variety of enzymes available in nature continues to make them highly attractive to that particular field of analytical chemistry.

Since the number of original papers describing applications of the various analytical aspects of enzyme–substrate reactions is large, especially in the biosensor and pharmaceutical area, this review intends to give an introduction into the state of art of the involved principles, taking recent applications as examples. Whenever possible cross-references to reviews of certain typical and important areas of application will be given.

2. Kinetic and thermodynamic consideration of enzyme reactions

The interaction within the bioaffinity complex is usually non-covalent, although the reversible formation of certain high-energy covalent bonds is also known. The reaction is reversible; thus an association and a dissociation reaction need to be considered.

Association reaction: $A + B \rightarrow AB$, $r_{as} = k_{as} c_A c_B$ (1a)

Dissociation reaction: $AB \rightarrow A + B$, $r_{dis} = k_{dis} c_{AB}$ (1b)

with r: reaction rate, k: rate constant, c_i: concentration of substance I.

Once the equilibrium has been reached, complex association and dissociation occur at equal speed, and the equilibrium binding constant (''affinity constant''), K, is defined as follows:

$$K = k_{as}/k_{dis} = c_{AB}/(c_A\, c_B) \quad (2)$$

High K values and low dissociation rate constants represent strong binding and stable complexes.

Enzymes present a special case, since they convert rather than only bind their substrates. For an enzyme reaction involving a single substrate and a single active unit (normal Michaelis–Menton kinetics) the following general reaction equation can be written:

$$E + S \leftrightarrows ES \dashrightarrow E + P \quad (3)$$

with E: enzyme, S: substrate, ES: enzyme–substrate complex, and P: product.

With the Michaelis–Menton constant, K_M, defined as:

$$K_M = (k_{\leftarrow} + k_{\rightarrow})/k_{\rightarrow}, \quad (4)$$

the rate of the enzyme catalyzed reaction, v, can be expressed by the Michaelis–Menton equation as a function of the constant, K_M, and the maximal possible catalytic rate, v_{max}:

$$v = v_{max}\, c_S/(c_S + K_M) \quad (5)$$

with v_{max} being equal to $k_{\rightarrow}$ multiplied by $[E_T]$, i.e., the total enzyme concentration in the system.

When K_M is small compared to c_S, v becomes equal to v_{max} and thus depends only on the total enzyme but no longer on the substrate concentration. If the opposite is the case and K_M is small compared to c_S, v depends linearly on both the enzyme and the substrate concentration. If, in addition, the enzyme concentration is kept constant, the catalytic rate will be a direct measure of the substrate concentration. Constant enzyme concentrations (or frequent recalibrations) are therefore necessary in systems, for example biosensors, which use an enzymatic reaction as analytical tool. Both, the final equilibrium or the on-set rate of the enzymatic reaction (kinetic method) can then be used to measure the substrate concentration. The former takes longer but usually yields more reliable results.

At last a distinction has to be made between homogeneous and heterogeneous enzymatic reactions. In the former case both reactants are in solution, while in the latter one of them, either the enzyme or the substrate is immobilized. Often the immobilization of a reactant is considered to create a somewhat ''artificial'' situation enforced by the requirements of the analytical system (biosensor, column, electrophoresis gel). However, it should be kept in mind, that a large number of enzymes are naturally ''immobilized'' for example in/on a cell membrane or a multi-enzyme complex. In these cases kinetic and thermodynamic date collected for the dissolved enzymes may be misleading.

An immobilization can have an impact on the reaction kinetics, but not necessarily so. Accessibility is of major importance and as long as it is granted, similar constants have been measured for the free and immobilized forms at least of small substrate molecules. Often this required immobilization of the enzyme via a spacer molecule. A parameter, which is often improved by an immobilization, is the stability of a given enzyme. While many of these protein molecules have half-lives of hours at room temperature, excellent preservation of the activity over months is reported for the same enzymes in immobilized form. The initial activity may be diminished, though, as a result of the immobilization. While changes in the enzyme structure are possible causes of this, a reduced accessibility of the active site is more likely the reason. In this context advances in the immobilization procedures towards a sterically controlled and optimized linkage are necessary.

A most important result of an immobilization is the increased influence of mass transfer effects, i.e., the transport by molecular diffusion of the substrate and product molecules to and from the enzyme. Mass transfer effects can usually be safely neglected for homogeneous enzyme reactions. They may become the rate-limiting step in heterogeneous reactions, where the total scheme thus can be represented as follows:

$$\begin{array}{ccccc} E + S_o & \leftrightarrows & ES & \dashrightarrow & E + P_o \\ \updownarrow & & & & \updownarrow \\ S_b & & & & P_b \end{array} \qquad (6)$$

S_b and P_b stand for the substrate and product concentrations in the bulk phase, whereas S_0 and P_0 represent the concentrations in the immediate vicinity of the enzyme.

A problem peculiar mainly to homogeneous analytical affinity separations is the occurrence of a secondary non-specific interaction, i.e., with the stationary phase in gel filtration or the capillary wall in affinity capillary electrophoresis. Many customary measures to prevent this (such as working at extreme pH or in the presence of certain buffer additives) cannot be taken, since they also exert an influence on the affinity interaction. Wall coatings seem to be the best solution in affinity capillary electrophoresis [2], while the introduction of repulsive charges on the stationary phase surface has been suggested for chromatography (most proteins carry a negative net-charge at neutral pH) [3]. However, such non-specific interactions do not necessarily have an adverse influence. Only when the interaction kinetics are influenced by the adsorption will the data be biased. As long as both the complex and the single enzyme interact to a similar extent the data are still valid.

In addition to the enzyme kinetics an investigation of the reaction thermodynamics may also yield important data, for example, in regard to the proposed interaction mechanism [4]. The equilibrium constant yields directly the free enthalpy of the interaction, ΔG_0:

$$\Delta G_0 = 2.303RT \log K \qquad (7)$$

with R: universal gas constant and T: temperature in Kelvin.

The enthalpy, ΔH, and the entropy, ΔS, can in turn be calculated from the van't Hoff plot of log K vs. $1/T$:

$$\log K = \Delta H/2.03RT + \Delta S/2.303R \qquad (8)$$

It is known that hydrophobic interactions tend to be entropy driven, while hydrophilic ones depend more on a favorable reaction enthalpy. A simple examination of the slope of the van't Hoff plot thus allows a statement concerning the hydrophilicity or hydrophobicity of the involved interactions.

In this context the interaction between concanavalin A and dextran as well as that between fibronectin and collagen were found to be enthalpy driven, i.e., hydrophilic. Consequently the affinity decreased with increasing temperature. The binding

between MOPC-315, a mouse myeloma immunoglobulin and di- and trinitrophenyl was found to be hydrophobic, i.e., in this case the affinity increased with increasing temperature [2]. Fig. 2 shows for four temperatures the affinity plots created for the interaction between MOPC-315 and trinitrophenyl and the corresponding van't Hoff plot.

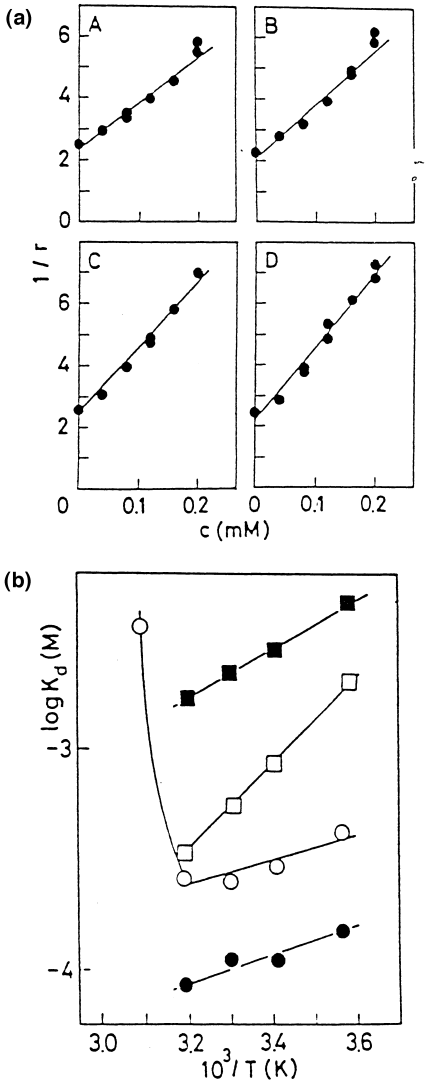


Fig. 2. (a) Affinity plot of interaction between MOPC-315 monomer and TNP ligand, obtained at 7°C (A), 20°C (B), 30°C (C), and 40°C (D). (b) Van't Hoff plot of the interaction between MOPC-315 monomer and TNP ligand (reproduced with permission from Ref. [95]).

3. Measuring principles in analytical affinity separations using soluble reactants

The stability of a given affinity complex determines to a large degree the type of analytical affinity separation for which it is suited. Only if the half-life of the complex is two- to three-orders of magnitude smaller than the time required for the analysis, t_{anal}, kinetic effect can be excluded and equilibrium be assumed [5]. If the ratio is only one- or two-orders of magnitude, a distortion of the analyte zone (''peak broadening'') must be expected. The half width of the peak could in principle be used for an estimate of the rate constants for complex formation and dissociation, mainly by applying computer simulations of various possible association/dissociation constants followed by a comparison with the experimental data.

For even more long-lived complexes the zone becomes to broad for an evaluation until for very stable complexes a situation is reached where the complex half-life is long compared to t_{anal} and the ratio of complex concentration to that of the individual reactants does not change during the analytical run. Such very stable complexes can again used in analytical affinity separations since the equilibrium constant may in this case be calculated from the ratio of the free to the bond ligand.

For most slab gels and chromatographic columns, t_{anal} is in the range of hours and many affinity complexes can be said to equilibrate fast enough. This may be different in the case of affinity capillary electrophoresis, where an electrophoresis time of only minutes is quite common.

The most important methods used to study biospecific interactions in solution are outlined below. Even in the case of the interaction of an enzyme with its substrate only the association/dissociation reaction is considered, i.e., in case of multiple ligand binding:

$$\mathrm{P} + n\mathrm{L} \leftrightarrow \mathrm{PL}n,\ r_{as} = k_{as}\, c_P c_L{}^n \text{ and } r_{dis} = k_{dis} c_{PLn} \quad (9)$$

with L: ligand, P: protein (enzyme).

Once the equilibrium has been reached, complex association and dissociation occur at equal speed and the general equilibrium constant, K, is defined as:

$$K = k_{as}/k_{dis} = c_{PLn}/(c_P c_L{}^n) \quad (10)$$

More complex algorithms exist for allosteric enzymes, where the affinity constant is not constant [6].

Data analysis is usually based on the Scatchard plot [7], i.e., the distribution isotherm, where the amount of bound ligand, L_b, per protein is expressed as a function of unbound fraction, L_f:

$$R = L_b/P = \sum(nKL_f)/(1 + KL_f) \quad (11a)$$

or for single site interaction:

$$R/L_f = nK - KR \quad (11b)$$

A plot of R/L_f vs. R has for a 1:1 complex a slope of $-K$ and an intercept of nK.

For the construction of this plot it is necessary to measure the bound as a function of the free ligand concentration without disturbing the equilibrium. Methods to achieve this are often size selective, for example, equilibrium dialysis, ultracentrifugation, filtration, or size-exclusion chromatography. The chromatographic methods have been developed into several variants, many of which can also be used in gel and capillary electrophoresis. However, since they have mostly been developed for application in connection to size-exclusion chromatography, certain assumptions are usually made. Since the ligand is usually a small molecule compared to the proteins, a similar migration speed for the protein and the protein–ligand complex is assumed, which is concomitantly taken to be higher than that of the free ligand. This may not be the case, for example, in capillary electrophoresis, where migration depends largely on the mass to charge ratio. The methods outlined below can still be applied, however, even when the ligand moves faster than the protein/protein–ligand complex zone, the elution order of the peaks will just be opposite from the indicated one.

3.1. Zonal elution/migration

This method calls for an incubation of a given amount of protein with a varied amount of ligand. When the equilibrium is reached, the amount of free ligand is quantified. The distribution isotherm can be directly constructed from these data. For this method it is necessary that the half-life of the complex be large compared to the time required for analysis.

An interesting variant of this method, which is applicable whenever the complex dissociation falls within the time required for the analysis, is the peak splitting method [8,9]. The method was originally developed for chromatographic applications but can in principle also be transferred to (capillary) electrophoresis. In chromatography it is usually carried out on an ISRP (internal surface reversed-phase) column. The reversed-phase surface is located only inside the pores of these stationary phases and conditions should be chosen thus that this volume is not accessible to the protein/protein–ligand complex molecules. The ligand on the other hand has access and is retained. In the ensuing chromatogram two peaks are detected, one corresponding to the original amount of free ligand (plus perhaps some weakly bond ligand molecules dissociated early on). Ligand molecules dissociating during the analysis cause the second, often rather broad peak, which elutes first. In theory both the bond and the free ligand concentration can thus be accessed in a single run. Whether or not the method is suitable for a given system must be assessed in each individual case.

3.2. Hummel–Dreyer method

In this method either the ligand or the protein is dissolved in the liquid phase [10,11]. A small sample is injected, which contains in addition a known amount of the respective other reactant. If the migration speeds of the protein–ligand complex and the free protein are similar and faster than that of the unbound ligand, then the protein/protein–ligand complex will be separated from the free ligand in the sample plug, Fig. 3a. Since the total ligand concentration in the sample was identical to the ligand concentration in the buffer, the zone of the former sample plug will be lower in ligand concentration due to the fact that a certain amount has become bound in the complex and thereby removed. Concomitantly will the protein/protein–ligand complex zone appear to be enriched in ligand. If the detector registers the ligand concentration, both a negative peak corresponding to the free ligand concentration and a positive peak corresponding to the bound ligand concentration will be seen in the detector trace, Fig. 3b.

In the original Hummel–Dreyer method, the cali-

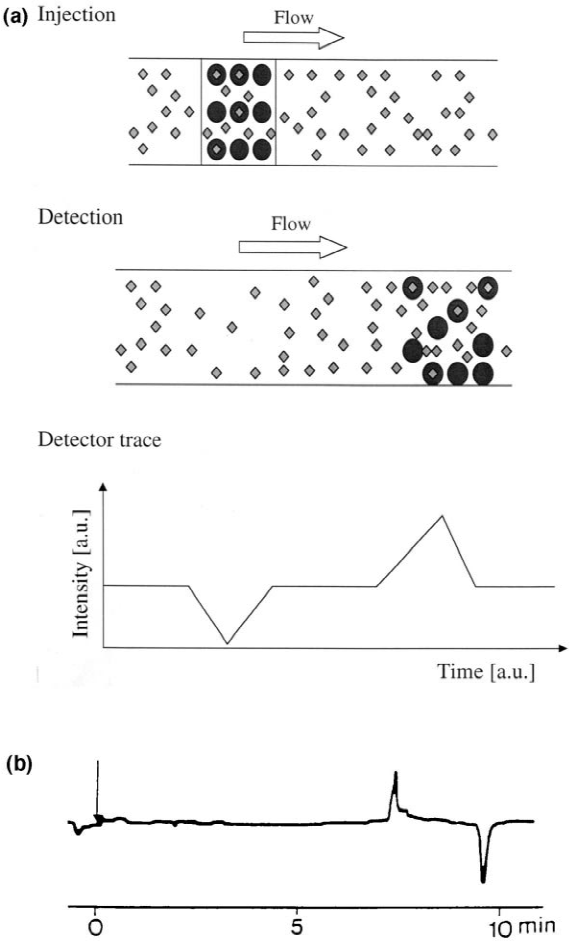


Fig. 3. (a) Principle of the Hummel–Dreyer method Figure. (b) Electropherogram obtained for the investigation of the interaction of the protein bovine serum albumin (BSA) with a small drug molecule (Warfarin) by the Hummel–Dreyer method (reproduced with permission from Ref. [96]).

bration for the free ligand peak was done by adding a known surplus of ligand to the sample and determining the ligand concentration necessary to ''fill up'' the negative peak. This is clearly time and reagent consuming. In the modified version according to Pinkerton and Koeplinger [12], only two injections are necessary one containing the protein sample and the other only buffer. The bound ligand concentration, L_b, is calculated according to:

$$L_b = c_L(A_s - A_e)\,/A_e \qquad (12)$$

with c_L ligand concentration in the buffer, A_s: area of the sample peak, and A_e: area of the buffer peak.

3.3. Vacancy peak method (equilibrium saturation)

In this method the buffer contains both a defined protein and a defined ligand concentration [13]. Then a ''sample'' containing only buffer is introduced. As migration begins, the protein/protein–ligand complex molecules will enter the ''empty'' zone faster than the free ligand ones, while at the same time free ligand molecules will be left behind in respect to the protein/protein–ligand complex ones at the other end of the zone, Fig. 4. Once the two ''borders'' meet, equilibrium is reestablished. The detector trace will show two negative peaks, the first one corresponding to the bound and the second one to the free ligand concentration, Fig. 4b. Usually the free ligand peak is less distorted and used for evaluation. The calibration of this peak is identical to that described for the Hummel–Dreyer method.

3.4. Frontal analysis

For this method a rather large sample consisting of the protein, the ligand, and the complex dissolved in buffer is injected into a system filled with pure buffer [14]. Once migration starts, the protein/protein–ligand complex molecules move at equal speed ahead of the bulk zone, while the free ligand molecules are ''left behind'', Fig. 5a. In the detector trace two plateaus will be seen, the first one corresponding to the protein/protein–ligand complex concentration and the second one to the free ligand concentration, Fig. 5b. From measurements of the free ligand concentration, L_f, for samples containing constant amounts of protein but varied amounts of ligand, the distribution isotherm is constructed. L_f is calculated from the ratio of the plateau height in the sample divided by the plateau height for a sample containing just the ligand in the given concentration (i.e., in the absence of binding by the protein).

The method can also be applied if the protein and the complex differ in migration time, then a third plateau is observed if the detector is capable of detecting the free protein [15]. The evaluation of the data remains the same. Sample volumes are large in chromatographic frontal analysis (several milliliters

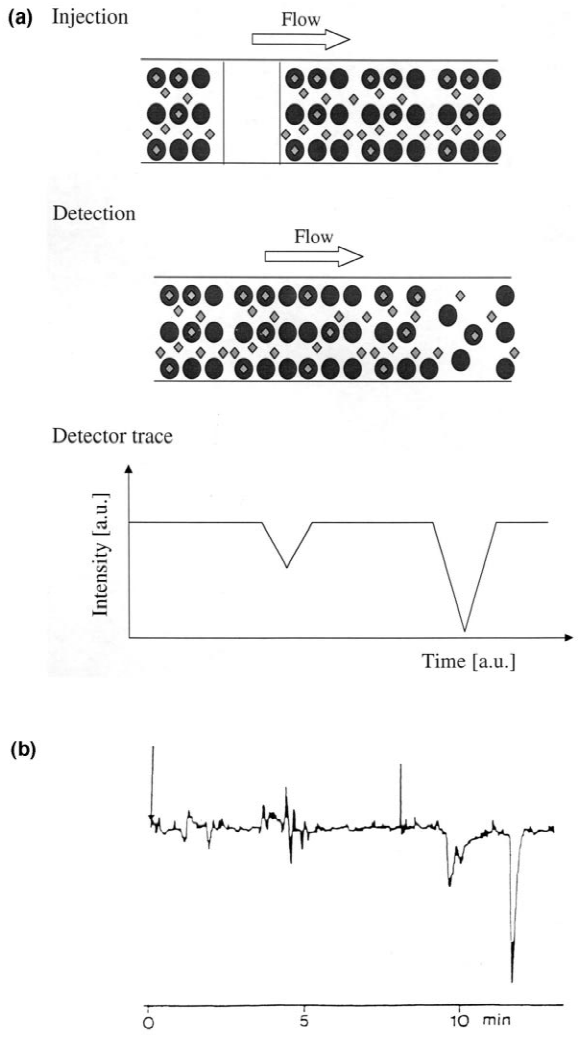


Fig. 4. (a) Principle of the vacancy peak method. (b) Electropherogram obtained for the investigation of the interaction of the protein bovine serum albumin (BSA) with a small drug molecule (Warfarin) by the vacancy peak method (reproduced with permission from Ref. [96]).

at least). This is one area where the inherently smaller sample volumes of capillary electrophoresis may constitute a definite advantage.

3.5. Mobility/retention shift analysis

In the case of quickly dissociating complexes, a change in migration/retention time may be used for analysis. The interaction of the ligand with the

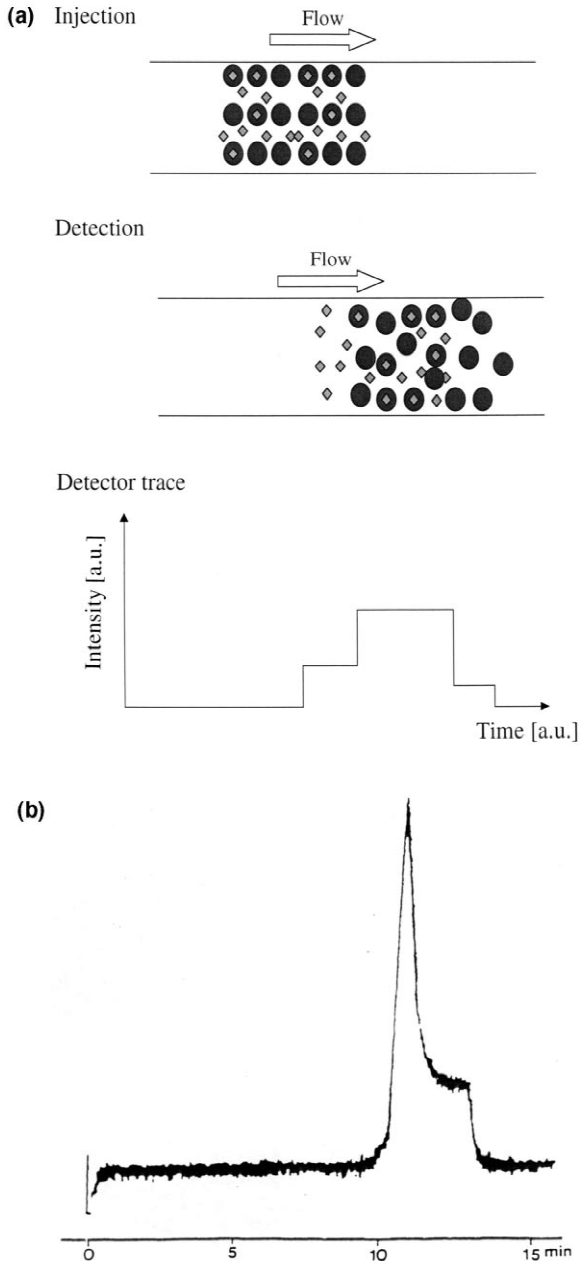


Fig. 5. (a) Principle of frontal analysis. (b) Electropherogram obtained for the investigation of the interaction of the protein bovine serum albumin (BSA) with a small drug molecule (Warfarin) by frontal analysis (reproduced with permission from Ref. [96]).

protein must result in a distinct change in the elution time or volume. How this can be brought about, depends on the individual system. In size-exclusion chromatography, the free ligand and the protein bond ligand obviously differ in retention time. The same may be the case in gel electrophoresis. However, in this case the binding of a small but highly charged ligand to a low charge density protein will change the charge but not the mass, which also influences the migration. In capillary electrophoresis it is mainly the latter effect, i.e., the increase in charge density by the binding of the small ligand, which is exploited.

In the mobility shift assays the protein is dissolved in the buffer and a small amount of ligand is injected (or vice versa). The migration time of the ligand is measured for a number of protein concentrations. When t_L is the migration time for the free ligand (i.e., in the presence of a protein concentration of 0) and $t_{L,max}$ the migration time limit observed for very high protein concentrations, the experimentally observed migration time $t_{L,ex}$ is related to the bound ligand concentration, c_{Lb}, as follows:

$$c_{Lb}/c_{P,tot} = (t_{L,ex} - t_L)/(t_{L,max} - t_L) = \Delta t_L/\Delta t_{L,max} \quad (13a)$$

For a monovalent complex (the method has not yet been extended to multivalent complexes) the following relation exists to the binding constant, K:

$$\Delta t_L/c_P = K(\Delta t_{L,max} - \Delta t_L) \quad (13b)$$

K can thus be determined from the Scatchard plot: $\Delta t_L/c_P$ vs. $(\Delta t_{L,max} - \Delta t_L)$.

Fig. 6 shows how the migration time of carbonic anhydrase from bovine erythrocytes changes with

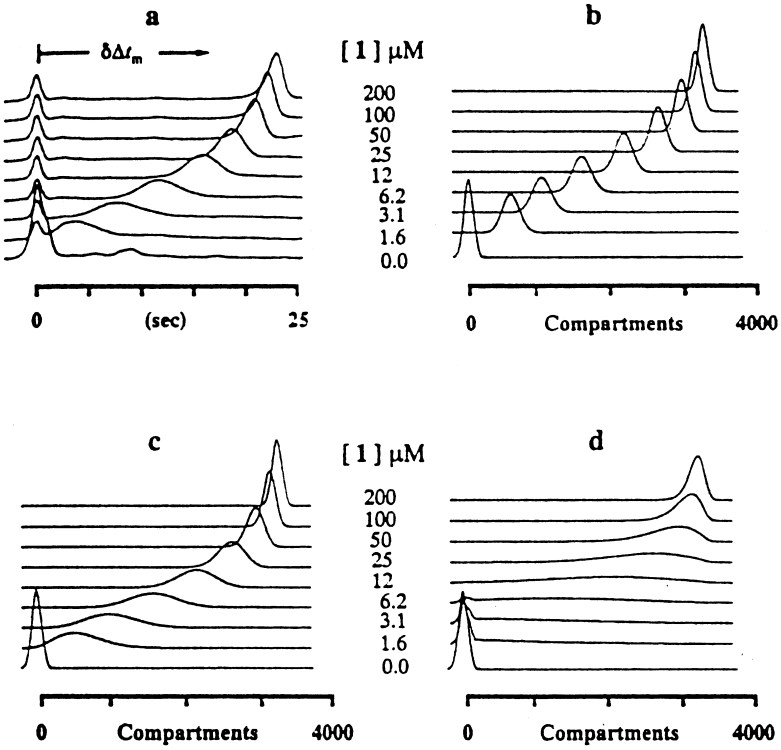


Fig. 6. Mobility shift observed when carbonic anhydrase from bovine erythrocytes is analyzed by affinity capillary electrophoresis in the presence of increasing concentrations of a charged affinity ligand. The non-mobile peak is horse heart myoglobin (Marker). The series in (a) was obtained experimentally, (b, c and d) were simulated assuming different association and dissociation rate constants. Clearly the values used in case c (k_{as}: $1.5 \cdot 10^4$ M^{-1} s^{-1}, k_{diss} 0.1 s^{-1}) correspond best with the experimental results (reproduced with permission from Ref. [16].

increasing concentrations of a charged ligand in affinity capillary electrophoresis [17].

4. Measuring principles in analytical affinity separations using immobilized reactants

The use of immobilized affinity ligands has revolutionized preparative bioseparation and the term affinity chromatography is currently used almost exclusively to denote a preparative technique. However, analytical affinity chromatography and a number of related gel electrophoresis techniques should not be neglected when searching for a method to investigate protein–ligand interactions. A clear advantage of the heterogeneous assay is the possibility to re-use the immobilized reactant.

The principles employed in analytical affinity chromatography are similar to those used for soluble reactants. If rapid equilibration allows an isocratic elution of the analyte, the elution time can be directly used to calculate the affinity constant. Breakthrough curves may be used for the same purpose. However, analogs of the vacancy peak and the Hummel–Dreyer method are also known.

Perhaps the easiest type of analytical affinity chromatography is the frontal affinity analysis (break through curve), where the sample containing a known concentration of the analyte is pumped continuously through the column. Given fast association/dissociation kinetics, the turning point of the breakthrough curve can be related to the concentration of the analyte, c_L, the concentration of active binding sites in the column, c_B, and the equilibrium constant for the interaction, K. If the analyte binds to a single type of binding site, the following equation may be used:

$$1/c_{Lb} = 1/(Kc_B c_L) + 1/c_B \quad (14)$$

with c_{Lb}: ligand concentration retained on the column at the turning point of the breakthrough curve. The equation can be modified to allow the investigation of multi-site interaction or competing ligands [1].

The development of a theoretical model for affinity chromatography involving enzymes requires the combination of enzyme kinetics with chromatographic theory. Since gel filtration (size exclusion) is by far the most popular of the chromatographic techniques used, the approach developed by Chaiken and his group will be outlined here [17,18]. According to this theory, the interaction of the enzyme with the immobilized ligand depends on the distribution of the enzyme between the mobile and the stationary phase on the one side (chromatographic theory) and the dissociation constant of the enzyme–ligand complex on the other (enzyme kinetics), Fig. 7.

$$E \overset{K_c}{\leftrightarrow} \underline{E} + LM \overset{K_e}{\leftrightarrow} E\text{-}LM, \quad (15)$$

with E: free enzyme, $\underline{E}$: intraparticular enzyme, LM: coupled ligand, and E–LM: enzyme–ligand complex.

The chromatographic equilibrium constant, K_c, is defined as:

$$K_c = c_{\underline{E}}/c_E \quad (16a)$$

The enzymatic complex dissociation constant, K_e, is defined as:

$$K_e = c_E c_{LM}/c_{E-LM} \quad (16b)$$

For a gel filtration column the elution volume, V_e, is linked by the following equation to the void volume, V_0, and the internal stationary phase volume, V_i:

$$V_e = V_0 + K_c V_i \quad (17)$$

The interaction between the enzyme and the immobilized ligand can be incorporated into this equation by defining a new equilibrium constant K_c', which takes the additional interaction between the enzyme and the stationary phase into account:

$$K_c' = (c_{E-LM} + c_{\underline{E}})/c_E \quad (18)$$

The experimentally observed elution volume, V_e' thus is:

$$V_e' = V_0 + K_c' V_i \quad (19)$$

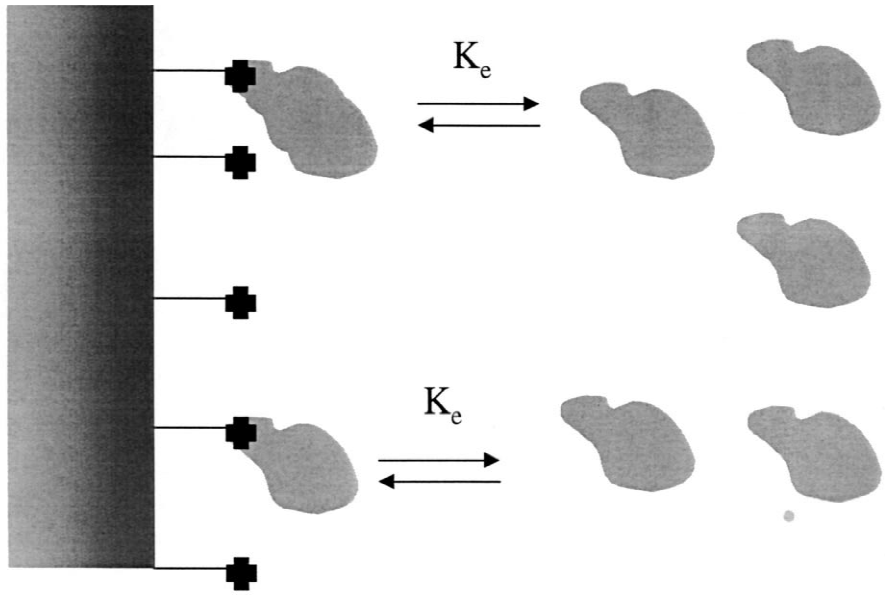
and combination of Eqs. (16b), (17), (18) and (19) yields:

$$V_e' = V_e + (V_e - V_0)c_{LM}/K_e \quad (20a)$$

or

$$1/(V_e' - V_e) = K_e/[c_{LM}(V_e - V_0)] \quad (20b)$$

The increase in retention volume due to the inter-



$$1 / (V_e' - V_e) = K_e / (c_{LM}(V_e - V_o))$$

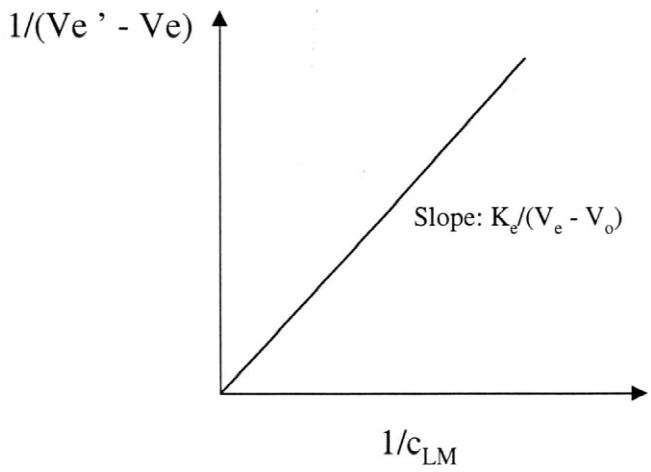


Fig. 7. Principle of analytical affinity chromatography based on isocratic elution.

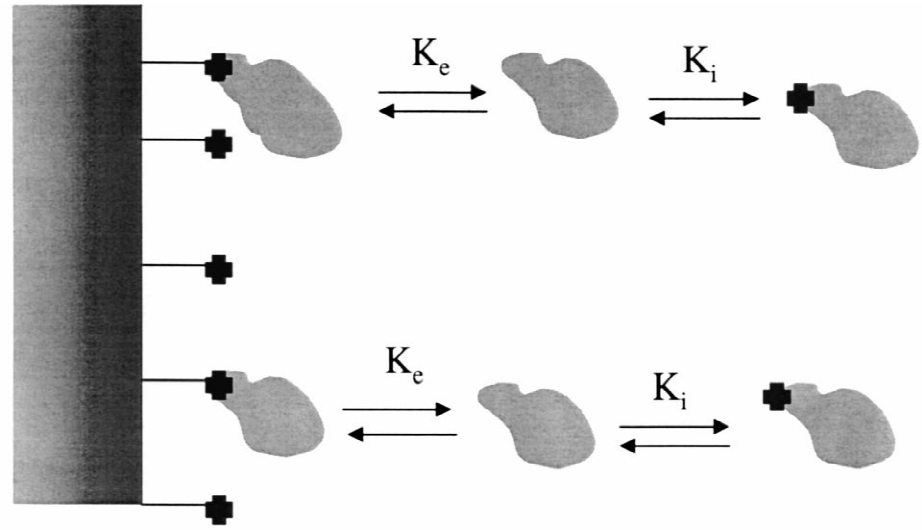
action is thus proportional to the concentration of the immobilized ligand c_{LM} and inversely proportional to the dissociation constant of the complex, K_e.

Since in many cases an isocratic elution of the tightly bound enzyme is not practical, Dunn and Chaiken suggested the modification of the equilibrium by adding a soluble ligand or competitive inhibitor to the mobile phase.

$$\begin{array}{ccccc} & K_c & & K_e & \\ E & \leftrightarrow & \underline{E} + LM & \leftrightarrow & E\text{-}LM \\ + & & + & & \\ I & & I & & \\ \downarrow K_I & & \downarrow K_I & & \\ EI & & \underline{E}I & & \end{array}$$

where K_I and $K_{\underline{I}}$ represent the equilibrium constant of the enzyme inhibitor complex outside and inside the beads. Thus a new equilibrium is established, Fig. 8, and Eqs. (20a) and (20b) change to:

$$V'_e = V_e + (V_e - V_0)(c_{LM}/K_e)[K_I/(K_I + c_I)] \qquad (21a)$$



$$1/(V_e\,'\text{-}V_e) = 1/(V_e\text{-}V_o)\ (K_e/c_{LM}) + 1/(V_e - V_o)\ (K_e/c_{LM})\ (1/(K_I c_I)$$

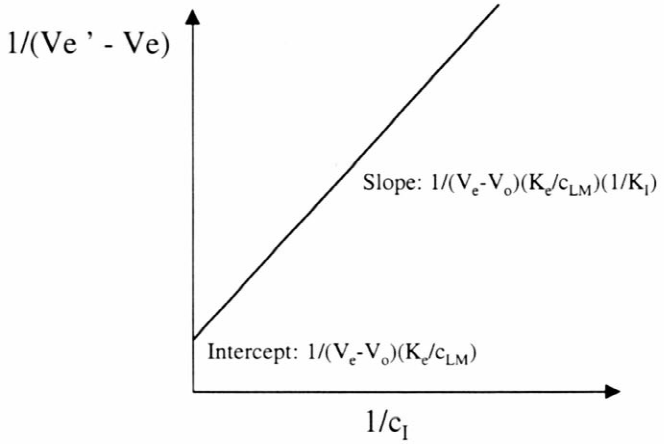


Fig. 8. Principle of analytical affinity chromatography based on competitive elution.

and

$$1/(V'_e - V_e) = 1/(V_e - V_0)(K_e/c_{LM}) + 1/(V_e - V_0)(K_e/c_{LM})[1/(K_I c_I)] \quad (21b)$$

Single site competition will give a linear plot for $1/(V'_e - V_e)$ vs. c_I with a slope of $1/(V_e - V_0)$ $(K_e/c_{LM})(1/K_I)$ and an axial intercept of $1/(V_e - V_0)(K_e/c_{LM})$. The ratio of the intercept to the slope yields K_I, while the intercept itself can be used to calculate K_e, if c_{LM} and V_e are known.

Since the ligand concentration is somewhat difficult to assess exactly (e.g., due to steric occlusion), improvements of the above outlined reasoning have been suggested [19,20].

Analytical affinity chromatography is generally performed in the linear region of the interaction isotherm, i.e., only small amounts of protein or ligand are injected. The method has been widely used to investigate the influence of various parameters on the binding strength or for fast screening, e.g., of putative drugs [1,19,21,22]. However, care must be taken to mirror ''physiological conditions'' to some extent, else the measured values may not be representative. The possibility to investigate several substances simultaneously by recording a whole ''chromatogram'' is of special advantage in this respect. It has become especially interesting in connection to the investigation of quantitative structure retention relationships (QSRRs), where the effect of slight variation of the ligand's molecular structure on the binding strength is investigated [23,24]. Another question easily addressed by the isocratic elution approach is that of a possible displacement of a ligand from the protein-binding site by another.

Besides equilibrium constants, rate constants (association, dissociation kinetics) are also accessible by analytical affinity chromatography [25,26]. The dissociation rate constant, k_d, can be determined from the van Deemter plot, since it is linked to the stationary mass transfer contribution, H_s, to height of a theoretical plate by the following formula:

$$H_s = (2uk')/[k_d(1 + k')^2] \quad (22)$$

with k': capacity factor $[= (V'_e - V_0)/V_0]$ and u: linear flow velocity of the mobile phase.

Once k_d and K are known k_a can be calculated.

The use of immobilized reactants for the analysis of biological interactions is also very common in classical gel electrophoresis [27]. Biologicals may either be immobilized or simply physically entrapped in the gel matrix.

5. Analytical chromatography based on enzyme–ligand interactions

5.1. Methods using immobilized reactants (analytical affinity chromatography)

The majority of the recent applications of analytical affinity chromatography seem to be in the area of drug protein interaction. However, enzyme–substrate complexes and their reaction mechanisms may also be investigated by this method [28]. Usually a coenzyme such as ATP or NAD(P) is immobilized on a matrix using established techniques [29]. Coenzyme A has also been suggested for this purpose [30].

Citrate synthase catalyses the reaction of acetyl coenzyme A and oxaloacetate to coenzyme A and citrate. The binding of the enzyme to columns activated with either acetyl coenzyme A or coenzyme A as well as various derivatives thereof known as inhibitors of the enzyme was investigated by Limbach and Schmidt [30]. The coenzyme A was immobilized either through the SH or the adenine moiety. The eluting power of the coenzyme and the influence of oxaloacetate on the binding were studied. It was possible to make deductions concerning the importance of various molecular structures within the coenzyme molecule for binding. Kinetic concepts proposed in the literature in regard to the binding sequence could be verified, since the enzyme was bound by acetyl coenzyme A even in the absence of oxaloactetate and oxaloacatate was not capable of eluting the enzyme from a coenzyme A column or influence the elution power of coenzyme A and its analogs. It did, however, enhance the elution power of acetyl coenzyme A to a large degree. Since coenzyme A is involved in many enzymatic reactions, the method has a potential for broader application.

Brodelius and Mosbach [31] chromatographed a

number of lactate dehydrogenases with known NADH affinity constants on an N^6-(6-aminohexyl)-AMP-agarose column. The known affinity constants were plotted against the NADH concentration necessary to elute the respective dehydrogenase and the plot used to determine the NADH affinity of so far uncharacterized dehydrogenases. In a similar approach Junowicz and Paris [32] used a Sepharose 4B column activated with a modified glycoside substrate to separate glycosidase mixtures that were difficult to separate otherwise. Elution and separation was possible with gradients of the respective substrates or inhibitors. The authors stress the preparative potential of the method, however, an analytical application seems also possible. O'Carra and Barry [33] used analytical affinity chromatography to investigate the binding order of substrates to lactate dehydrogenase. They confirmed NADH as leading and pyruvate as trailing substrate, by investigating the effect of the pyruvate analog oxamate. Lakhiari et al. used analytical affinity chromatography to investigate the activity and the interaction mechanism of insulin and *N*-acetylneuramic acid as a function of various physico–chemical parameters [3].

Certain synthetic textile dyes are known to bind to a large number of proteins. The exact nature of the interaction can vary, however, the usually observed high affinity to pyridine nucleotide-dependent enzymes seems to be due to analogous structures in the two molecule classes, i.e., the dyes and the coenzymes. Thus textile dyes have repeatedly used to investigate enzyme reaction mechanisms by analytical affinity chromatography. Clonis and Lowe [34] used analytical affinity chromatography to elucidate the interaction of triazine dye analogs of certain coenzymes such as ATP, NAD(P) and acetyl coenzyme A. The goal of their studies was the optimization of the respective preparative application of these dyes. However, much insight in the involved binding mechanisms was gained, which in later years formed the foundation for the systematic development of biomimetic dyes. Liu et al. [35] used frontal and zonal chromatography, Fig. 9, as well as a static equilibrium method for the quantitative investigation of the interaction of rabbit muscle lactate dehydrogenase and immobilized reactive blue 2. It was found that each dye molecule binds one enzyme molecule and that binding takes place at the NADH binding

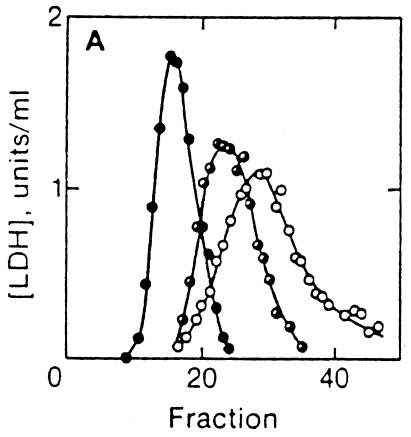


Fig. 9. Zonal analysis of the interaction with rabbit muscle lactate dehydrogenase with reactive blue 2 immobilized on Sepharose CL 6B. ○: No NADH, ◐: 0.9 μ*M* NADH, ●: 6 μ*M* NADH. 100 μl of a 1.2 μ*M* enzyme solution was applied in each case (reproduced with permission from Ref. [35]).

site. The analysis was carried out both for the pure enzyme and in the presence of NADH as competing ligand. Results were analyzed by Eqs. (20) and (21) (a and b), respectively. The assumption of a monovalent interaction underlying both equations was found verified in both cases. NADH was found to compete for the same binding site with the dye. In the static measurements the enzyme was brought into contact with the support and the concentration remaining in the supernatant after 2 h was determined. Values determined by the latter method were virtually identical to those obtained chromatographically.

The possibility that immobilized reactants may actually mirror the native form of the involved enzyme was already mentioned. This concept if fully developed in immobilized proteoliposome affinity chromatography (IPAC), where the affinity gel is prepared by steric immobilization of proteoliposomes containing a reconstituted membrane protein into rigid gel beads by freeze–drying, Fig. 10. The lipid environment and the steric immobilization favor retention of protein activity. The method was used by Yang and Lundahl [36] to investigate the interaction of a human red cell glucose transporter with a transport inhibitor and the transported solute, D-glucose, Fig. 11. The effect of various physico–chemical parameters was studied. Equilibrium con-

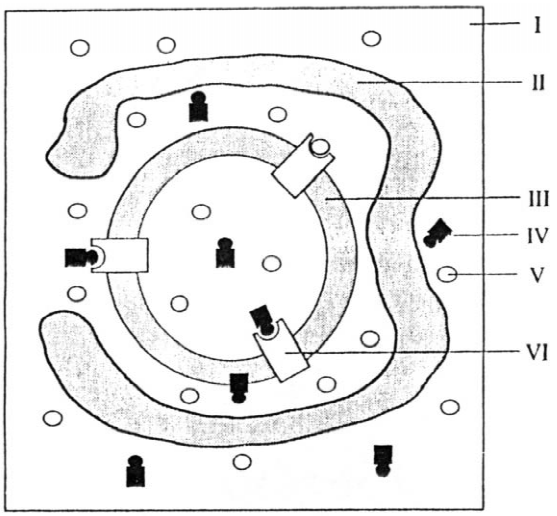


Fig. 10. Schematic presentation of the IPAC (immobilized proteoliposome affinity chromatography) principle. I: Gel bed, II: gel bead matrix, III: lipid bilayer of sterically immobilized proteoliposome, IV: interactant applied in a narrow zone, V: competing solute present in the eluent, VI: reconstituted membrane protein (reproduced with permission from Ref. [36]).

stants similar to those obtained with conventional methods were calculated. An advantage of the chromatographic method was that the amount of active protein (binding sites density) could be determined simultaneously without destroying the beads.

DNase (desoxyribonuclease I) is an endonuclease that hydrolyses DNA non-specifically. Recombinant human DNase was, for example, used as therapeutic in the treatment of cystic fibrosis. The enzyme exhibits considerable charge heterogeneity due to a complex glycosylation pattern. In addition, deamidation of certain labile asparagine residues is possible. Since at least one of the asparagines may be important to the biological activity of the enzyme, the quantification of the deamidated fraction is important for quality control. Normally the charge difference between the native and the deamidated form of a given protein can be exploited to determine this ratio. In case of the DNase, this difference is hidden by the natural charge heterogeneity. Instead protein sorting by analytical affinity chromatography was used [37]. A non-hydrolyzable double-stranded oligonucleotide analog of the DNA substrate was synthesized and immobilized. The column was not only able to resolve the native and the deamidated form of the recombinant DNase, but also the two isomeric forms of the deamidation product. The authors saw this as a possible contribution to the elucidation of the biochemical interaction between variants of an enzyme and its substrate.

5.2. Methods using soluble reactants

Normally the investigation of the interaction between an enzyme and its substrate in solution is not feasible by chromatographic means, since the turn-

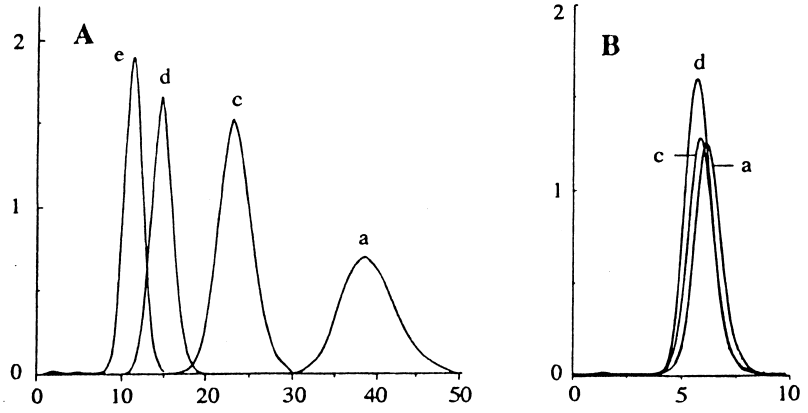


Fig. 11. Retardation of cytochalasin B (CB) upon IPAC runs on 1-ml gel beds with immobilized proteoliposomes with non-purified glucose transporter Glut 1 (A) or protein-free liposomes (B). The gel beads contained 1.1 mg of polypeptides/ml and 72 μ*M* of EPLs (egg phospholipids) in case A and 55 μ*M* EPLs in case B. Run a was without glucose, runs c, d, and e with 0.05 *M*, 0.2 *M* and 0.5 *M* glucose, respectively (reproduced with permission from Ref. [36]).

over rate is too high. This may be different, however, in the case of some large substrates such as DNA and polysaccharides. As pointed out above, the separation of free and bond reactants is required in an investigation of the interaction between soluble molecules. In the majority of the cases size-exclusion columns have been used to that end, but other approaches are also possible.

Takahashi and Hagmar used ion-exchange chromatography to investigate the interaction of RecA, a protein required for general genetic recombination in *Escherichia coli*, and double stranded DNA [38]. In this case the dissociation of the complex could be slowed down by the use of a non-hydrolyzable ATP co-factor analog (ATPγS). Both free DNA and free protein could be measured by this method, Fig. 12, while most alternative assays such as gel shift and filter binding would have quantified only the free DNA concentration. Thus less assumption had been made. The determination of the free protein concentration in addition to the DNA concentration also allowed the determination of binding parameters such as the association constants. Since high-performance chromatography was used, the reliability and reproducibility of the measurements was very high. Per RecA monomer 3 base pairs were bond. The binding exhibited cooperativity and only 1:1 complexes between the two molecules were formed.

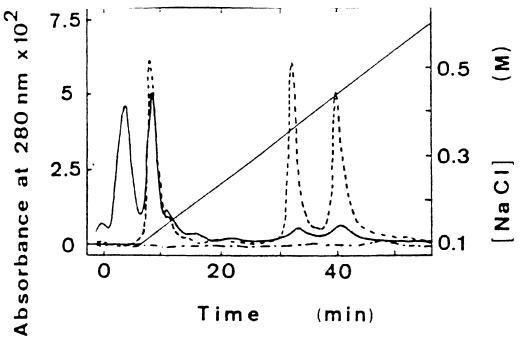


Fig. 12. Elution by NaCl gradient of a mixture of 30 μ*M* double stranded DNA, 10 μ*M* RecA protein, and 50 μ*M* ATPγS charged immediately after mixing (- - -) or after 30 min of incubation at 25°C (_) on a DEAE 5PW column (reproduced with permission from Ref. [38].

6. Affinity electrophoresis

Affinity electrophoresis is the broadest sense of the word denotes all techniques in which some kind of biospecific interaction between an electrophoresed component and another component (ligand) present in the medium occurs. The interaction results in a change in electrophoretic mobility of the electrophoresed substance compared to its mobility in the absence of the specific ligand in the medium. The observed effect should be most pronounced when the two components the ligand and the protein are of similar size or when the ligand is highly charged.

6.1. Affinity electrophoresis in conventional (slab) gels (AEP)

In the case of slab or rod gels, affinity electrophoresis has the additional advantage that no sophisticated instrumentation is necessary save for that, which is already routinely found in most biochemistry labs. From very early on, the method was used to detect ligand-binding components in a sample (e.g., Refs. [39–43]) or to determine a binding heterogeneity [43,44]. It is also suitable for quantification of the ligand binding components, or for a quantitative study of the protein–ligand complex formation, e.g. changes resulting from various treatments [45–47].

In the case of immobilized ligands the following basic equation may be used:

$$1/(d_0 - d) = K_e/d_0 c_{\underline{L}M} + 1/d_0 \quad (23)$$

with d: migration distance in the affinity gel, d_0 migration distance in the control gel.

In analogy to analytical affinity chromatography, the concomitant interaction with a second soluble molecule, I, can be investigated based on Eq. (24):

$$d/(d_0 - d) = (K_e/c_{\underline{L}})(1 + c_I/K_I) \quad (24)$$

Certain assumptions are made in the application of this treatment: (a) the immobilization of the ligand is complete, i.e., the mobility of the protein ligand complex is zero, (b) the mobility of the protein–free ligand complex is identical to that of the protein, (c) the concentration of the protein is much lower than

that of either ligand, (d) complex formation and dissociation are rapid, (e) the protein contains single binding site, (f) all molecules of the immobilized ligand are equally accessible, (g) the influence of the electric field on either complex formation is negligible, (h) the gel material has no influence of on the complex formation.

The basic theory of affinity electrophoresis has reached a state were some deviations from the assumptions can be considered [27]. If the immobilization of the ligand is not complete and the protein–free ligand complex and the protein differ in migration speed, an apparent dissociation constant $K_{e,app}$ can be determined from Eq. (25) [48]:

$$1/(d_0 - d) = K_{e,app}/(d_0 - D)(1/c_{LM}) + 1/(d_0 - D) \quad (25)$$

with D mobility of the protein–''immobilized'' ligand complex.

Multiple but independent interactions as well as steric hindrance effect of ligand immobilization can also be incorporated into the data evaluation [49].

Quantitative assays for a sequence specific binding of proteins to DNA have allowed major advances in the understanding of gene regulation on a molecular level. Gel electrophoresis is particularly sensitive to changes in the mass/size (form) of the sample molecules. As such it has enjoyed a long tradition of being used for the analysis of protein–DNA complexes, since its first quantitative application by Garner and Revzin [50] and Fried and Crothers [51]. Compared to other analytical methods such as sedimentation, blotting, filter binding, and immunoassays, electrophoresis is characterized by high resolution, speed, and simplicity. No specific reagents such as antibodies are required. Even weak binding between proteins and DNA can be detected, in contrast to most other methods, which tend to require the formation of stable complexes. The gel matrix has in addition a stabilizing effect on the protein–DNA complexes, so little dissociation takes place during analysis. Raw extracts can be used, since electrophoresis allows combining a general separation step with the binding analysis. The latter is of especial importance for the discovery of new binding proteins from cell extracts.

Native gel electrophoresis (mobility shift) assays are used to obtain quantitative information about the size distribution, the equilibria and the kinetics of protein DNA interaction [52]. Most assays are based on a reduction of the mobility of DNA restriction fragments upon binding to the proteins. The optimal fragment size for this is 1 kilobase (kb) pairs. The simplest version of the assay required the addition of a small amount of DNA binding protein to a mixture of restriction fragments. The fragment binding the protein will be slowed down in the gel. Slow dissociation and quantitative separation of free and bond DNA is required for a successful analysis.

Besides analysis of crude extracts, the method has also been used for equilibrium and kinetic studies of protein–DNA interaction and binding interfering assays [53]. In cases where several proteins are involved in the regulation of a single gene, such an investigation may allow insights into the mechanisms, sequence, and effects of the protein binding. A general review of the various techniques is supplied by Hendrickson [54]. Ng et al. used the native polyacrylamide gel electrophoresis band-mobility shift assay to study the binding of synthetic oligonucleotides by DNA polymerase δ and proliferating cell nuclear antigen [55]. A model of the ordered sequential interaction of DNA polymerase δ, proliferating cell nuclear antigen, and DNA template-primers was proposed based on the electrophoretic data.

Application of gel electrophoresis to an investigation of the binding between proteins and RNA has been proposed by Konarska and Sharp [56]. Proteins involved in DNA replication and recombination have also been investigated [57,58]. Another major application is the study of structural elements required for protein DNA recognition. The binding domain of a transcription factor for the Xenopus 5S RNA gene was, for example, identified from a proteolytic digest by gel electrophoresis [59]. Again the raw digest could be used directly.

Other types of interaction involving large molecules can also be investigated by affinity gel electrophoresis, e.g., those between amylases and phosphorylases and immobilized starch and glycogen [60–63]. Nakamura et al. [64] used the method to investigate the influence of various parameters (tem-

perature, pH, additives) on the interaction between human plasma fibronectin and different types of collagen. An investigation of the thermodynamics of the interaction showed, that hydrophilic interactions play an important role. Sudo and Kanno used affinity electrophoresis to investigate the enzymatic properties of amylase produced in lung carcinoma [65]. In addition affinity gel electrophoresis using immobilized D-galactose has been used to investigate the interaction with an α-D-galactosidase [66] and a galactose oxidase [67] using various sugars as competing soluble ligands.

6.2. Affinity capillary electrophoresis (ACE)

Affinity capillary electrophoresis (ACE) has the advantage of being yet faster and requiring even less sample than the conventional types of electrophoresis. The basic principle of ACE again the altered electrophoretic mobility (migration shift assay) of the complexed species as compared to the free ligand [68,69]. No pure or even quantified sample is required in ACE, since the analysis is based on changes in the migration time rather than peak areas.

Since capillary zone electrophoresis is sensitive to both size and charge, ACE can also be used to investigate the binding of highly charged low molecular weight ligands and even simple ions [70] to an enzyme. Other methods suitable for the investigation of interactions between small ligands and proteins such as ultraviolet and fluorescence spectroscopy, equilibrium dialysis, nuclear magnetic resonance, differential scanning calorimetry, or analytical affinity chromatography usually required larger amounts of sample or labeled reagents. In principle the interaction of several proteins (or ligands) can be measured in parallel (drug screening, evaluation of peptide libraries) [71].

The binding of charged ligands can be quantified directly in ACE. For neutral ligands competition with a previously characterized charged ligand is necessary. The determination of the kinetic and equilibrium constants relies on the shift in migration times and the peak shape. Simulation of the protein mobility under conditions of ACE suggests that the experimentally obtained electropherograms can be explained in terms of a few variables: association and dissociation rates (and thus binding constant), ligand concentration, and relative mobility of the protein and the complex [16]. The binding and the rate constants can be determined from the same set of experiments.

The data evaluation is in principle similar to that described above for chromatographic separation of the various compounds. In ACE it is necessary to combine electrophoresis theory with enzyme kinetics. The mobility, μ, of a given species is in electrophoresis related to its net-charge, Z, and its hydrodynamic drag:

$$\mu = C_p Z/M^{\alpha} \tag{26}$$

with c_p: protein specific constant, M: mass.

Experimentally, μ is calculated from the following formula:

$$\mu = (L_t L_d)/V(1/t - 1/t_0) \tag{27}$$

With L_t: total length of the capillary, L_d: distance injector–detector, t: migration time protein, t_0: migration time neutral marker (EOF), V: applied voltage.

If we assume α to denote the degree of complex dissociation, $c_{L,tot}$ the total analyte (ligand) concentration, and c_P that of the binding protein concentration, the binding constant K for the interaction of L and P can be expressed as:

$$K = c_{LP}/c_L c_P = (1-\alpha)c_{L,tot}/\alpha c_{L,tot} c_P \tag{28}$$

or:

$$(1-\alpha)/\alpha = K c_P \tag{29}$$

If c_P is constant under experimental conditions, K can be determined from α as a function of c_P. Alternatively Eq. (29) can be expressed in mobility shifts:

$$(\mu_0 - \mu)/(\mu - \mu_\infty) = K c_P \tag{30}$$

with μ_∞: mobility of the fully ligand charged protein, μ_0 mobility of the pure protein, and μ: mobility of the protein–ligand complex at a given ligand concentration.

Rearrangement of Eq. (30) yields the usual equations suitable for Scatchard analysis:

$$\Delta\mu_p/c_L = K\,\Delta\mu_{p,max} - K\,\Delta\mu_p \tag{31a}$$

or, if migration times are substitutes for mobilities:

$$\Delta t_p / c_L = K\,\Delta t_{p,max} - K\,\Delta t_p \quad (31b)$$

with c_L: ligand concentration and $\Delta\mu_{p,max}$: difference in mobility between the pure protein and the fully ligand charged protein, $\Delta\mu_p$: change in mobility between the pure protein and the protein ligand complex in the presence of the given ligand concentration.

In applying Eqs. (31a) and (31b) it is assumed that the EOF does not change significantly with the addition of the ligand (else a marker may have to be used [72]), that the binding is monovalent, that equilibrium is achieved, and that neither the interaction of the protein and the ligand with the wall nor the presence of the electric field exert a significant influence on the binding. A peak broadening may sometimes be observed for intermediate migration times, which has been said to be due to the equilibration between the free protein and the complex, which differ in their mobilities. The peak width depends on the dissociation rate constant of the complex and the run time of the electrophoretic separation. The rate constants can be extracted from the electropherograms by modeling the separation for the electrophoretic parameters taking known quantities of the system, such as the equilibrium constant, the complex concentration and the relative mobilities of the protein and the protein complex into account [69].

Chu et al. [69] chose carbonic anhydrase as model protein and investigated the binding of this protein to various arylsulfonamides. The binding constants were similar to those determined by other methods. Reif and Freitag [73] used capillary zone electrophoresis for a simple investigation of the interaction between proteases, substrates, and the protease inhibitor α_2-macroglobulin, Fig. 13. Some deductions concerning the possible mechanisms of the enzymatic reactions were possible from the electrophoretic results.

Experiments using the capillary as a microreactor [74–76] have demonstrated that both enzymatic activity and the effect of ligand binding on the electrophoretic mobility of the involved enzyme can be investigated in such cases. Avila and Whiteside [74] used the technique, for example, to investigate the irreversible oxidation of glucose-6-phosphate by glucose-6-phosphate dehydrogenase and the reversible conversion of ethanol to acetaldehyde by yeast alcohol dehydrogenase. They assume that their technique could be used to examine samples for a certain enzymatic activity or concentration of substrate, as well as for the evaluation of molecules as potential substrates for certain enzyme-catalyzed reactions, or for studying enzyme-inhibitor interactions.

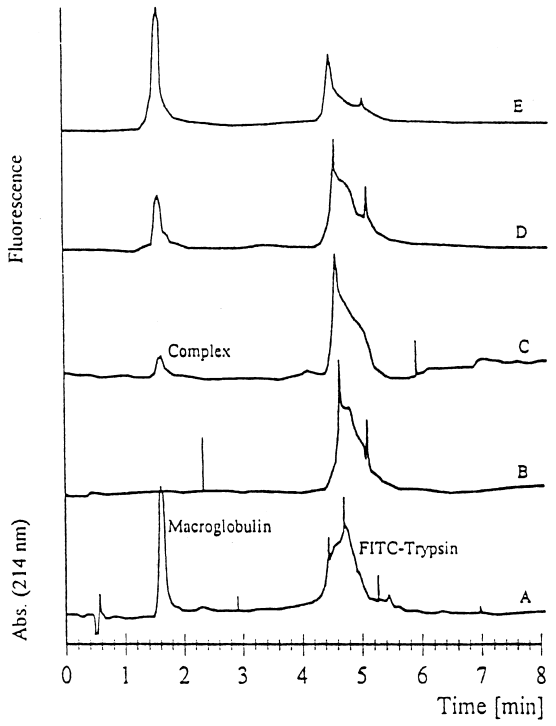


Fig. 13. Complex formation between α_2-macroglobulin and FITC-labeled trypsin. (A) α_2-Macroglobulin and FITC-labeled trypsin with UV detection, (B) α_2-macroglobulin and FITC-labeled trypsin with LIF (laser-induced fluorescence) detection and no incubation, (C) after 5 min incubation, (D) after 30 min of incubation, (E) after 60 min of incubation (reproduced with permission from Ref. [73]).

7. Bioanalytical systems

Analytical affinity chromatography, affinity capillary electrophoresis and the related techniques for obvious reasons tend to use biocapturing rather than bioconverting agents to provide the high selectivity typical for these analytical techniques. Thus enzyme–substrate interactions are rarely used in these systems, although their principal applicability should

never be neglected. Often an enzymatic activity first singles out a protein for closer inspection and the immobilized substrate may be a first way to isolate the protein and to investigate its function and structure. However, an entire area of analytical (bio-)chemistry is dominated by the bioconverting rather than the biocapturing agents and that is that of biosensors and other bioanalyzers. Bioanalytical systems and their role in bioanalysis will briefly be reviewed in the last section of this review.

7.1. Biosensors

Although different definitions exists, a biosensor is commonly described as an analytical device which uses a biological interaction for the specific recognition of the target molecule brought into close contact with a physico–chemical ''transducer'', which converts the biological reaction into an electronic output signal, Fig. 14. As pointed out above, the biological component can be a biocapturing agent such as an antibody, a receptor, or an oligonucleotide, but bioconverting agents such as enzymes are by far the preferred for the analysis of small molecules such as glucose (e.g., in medicine, biotechnology, food industry). For reviews, see Refs. [77–80]

In spite of the plentitude of enzymes known in nature, the number of basic reactions used in biosensor development is small. Perhaps for historical reasons, the first ''biosensor'' was an oxygen electrode covered by an enzyme membrane [81], oxidases are most often used, i.e., a reaction of the following general type:

$$\text{Substrate} + O_2 \xrightarrow{\text{oxidase}} \text{Product} + H_2O_2$$

Either the decrease in oxygen or the increase in hydrogen peroxide can be detected by a suitable transducer.

Dehydrogenases catalyze oxidation/reduction reactions involving NAD(P)H as coenzyme:

$$\text{Substrate} + \text{NAD(P)}^+ \xrightarrow{\text{dehydrogenase}} \text{Product} + \text{NAD(P)H}$$

The NAD(P)H can be detected either electrochemically (oxidation) or optically (fluorescence, absorbance). Often the pH shifts as a result of a dehydrogenase catalyzed reaction, which can also be used for quantification.

Hydrolases catalyze the hydrolysis of a compound

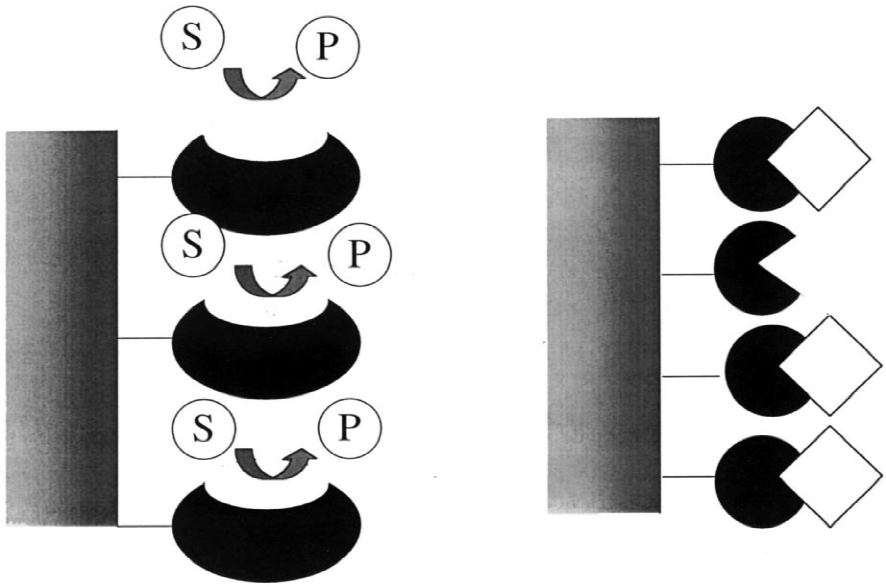


Fig. 14. Principle of a biosensor, left: bioconverting sensor, right: biocapturing sensor.

and often easily detected products are formed such as: H^+, OH^-, NH_3, etc. Protons are especially useful, since various pH-sensitive transducers are available.

Complex multi-enzyme systems are used, for example to ''shift'' the equilibrium of a first reaction to the product side or to obtain a directly measurable product, e.g.,:

$$\text{Disaccharide} + H_2O \xrightarrow{\text{disaccharidase}} \underline{\beta\text{-D-glucose}} + \text{monosaccharide}$$

$$\beta\text{-D-Glucose} + O_2 \xrightarrow{\text{glucose oxidase}} \delta\text{-gluconolactone} + H_2O_2$$

Coupled systems are also used to obtain a molecule that can be detected without bias caused by the sample matrix (a H^+ detection depends, for example, on the (changing) buffer capacity and pH of the matrix):

$$\beta\text{-D-Glucose} + O_2 \xrightarrow{\text{glucose oxidase}} \delta\text{-gluconolactone} + H_2O_2$$

$$H_2O_2 + \text{4-fluoroaniline} \xrightarrow{\text{peroxidase}} F^- + \text{polymeric products}$$

A fluoride-sensitive field effect transistor or other transducer can be used to quantify the F^-[82].

The physico–chemical transducers used for biosensors have to be able to correspond to the biochemical product [83]. Electrochemical (amperometric and potentiometric electrodes, field effect transistors) transducers are by far the most popular. Thermal (calorimeter, enzyme thermistor) and electrical (conductivity, capacitance) transducers have occasionally been used. The field of optical biosensors has enjoyed the most pronounced growth [84,85]. Compared to some of the others, optical transducers have certain advantages, including: easy operation, easy miniaturization (fiber optics), and no electric currents (explosion prove). Long distance measurements are possible. In addition, optical fibers are highly flexible, cheap, and sterilizable. Since the number of enzymatic reactions that result directly in the production of light are small, a pH or oxygen sensitive dye is often coimmobilized in the bioactive layer. The NAD(P)H fluorescence can also be used to follow reactions involving these co-enzymes.

The immobilization of the enzyme and various necessary coreactants is most important for the stability and reliability of the sensor both during storage and during actual use. Physical and chemical adsorption, entrapment into membranes, fibers, capsules, and polymer matrices, as well as cross-linkage are mostly used. Compared to affinity chromatography, covalent immobilization is less common in biosensor design. Presumably the necessity to activate the surface for a covalent immobilization is seen as handicap. However, the mechanical and chemical strength of the deposited biolayer would be improved by covalent attachment and perhaps even some of the current problems with maintaining sensor stability would be reduced by a covalent immobilization.

One reason for the attention biosensors enjoyed during the last decade is that the possible range of application seems so wide. Among others, biosensors seem suited for analysis in medicine (glucose sensor as part of the artificial pancreas, general analysis [86,87]), in biotechnology (process monitoring, quality control [88,89]), in environmental analysis [90], in defense [91], in agriculture and in the food industry [92]. Biosensors might even serve as artificial organs (noses) in robotics [93]. However, in spite of the efforts, the number of commercially successful system remains small and is concentrated almost exclusively into the medical area and for a single application, i.e., that of glucose monitoring. Even biotechnology, which has an acknowledged need for sophisticated monitoring devices is only reluctantly relying on biosensors for this purpose. The short life-time, the unsolved problem of how to interface the (usually non-sterile) sensor with the bioprocess and how to carry out the frequently necessary re-calibrations, as well as the limited analyte concentration range prevent application.

8. Conclusions

The interaction between enzymes and substrates plays an important role in analytical chemistry. Due to the extreme selectivity of the biological interaction, enzymes recognize and exclusively convert their substrates even in complex samples. In princi-

ple the interaction could be used for the investigation of enzyme kinetics in analytical affinity chromatography, affinity capillary electrophoresis and related techniques, especially when immobilized or large substrate molecules are used. However, such applications a rare compared to the utilization of immobilized enzymes as means to recognize and quantify their substrate in complex samples. The development of such ''biosensing'' devices is at present still handicapped by some inherent properties of the enzymes. As ''biopolymers'' their lifetime is limited even when immobilized to a stabilizing support, in comparison to that of some man-made polymers. Given the extensive research in the biosensor area including that of enzyme immobilization and preservation, a major breakthrough is required before such systems can become more than one-way articles. Maybe our insight in the structure function relation can be developed to a point where bioartificial hybrids can be used for detection purposes. The ''artificial enzymes'' created by molecular imprinting may present another option [94].

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