Journal of Chromatography, 644 (1993) 11–16
Elsevier Science Publishers B.V., Amsterdam

CHROM. 25 169

Salt-induced immobilization of proteins on a high-performance liquid chromatographic epoxide affinity support

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(First received August 4th, 1992; revised manuscript received April 6th, 1993)

ABSTRACT

The immobilization of thirteen proteins on a high-performance liquid chromatography epoxy affinity support was studied as a function of ammonium sulfate concentration. Without exception the fraction of protein immobilized at 20 h increased with increasing salt concentration at higher salt concentrations. Ten of the proteins were 95–100% immobilized in 20 h at ammonium sulfate concentrations ranging from 0.4 to 2.5 *M*. At lower salt concentrations the affinity support exhibited lower reactivity with all proteins. A kinetic model is proposed in which protein in solution is at equilibrium with a noncovalent protein–affinity support complex. The nucleophiles on the protein of the complex then react in a slow step with epoxy groups on the affinity phase. The dependence of immobilization on salt concentration is interpreted as a salt induced hydrophobic interaction on the equilibrium formation of the noncovalent protein–affinity matrix complex.

INTRODUCTION

We reported previously [1] that in studies of protein coupling to an epoxide-type, silica-based HPLC affinity support, the presence of relatively high concentrations of salt results in highly efficient protein immobilization at neutral pH. In that study the bound proteins, comprised of individual immobilizations of human albumin, α_1-acid glycoprotein and IgG immunoglobulin, retained good activity in the purification of their antibodies from antisera. The extent of coupling for two proteins (goat IgG and human α_1-acid glycoprotein) was examined and found to increase with salt concentration, and there was a large difference in the concentration of salt required to attain a given coupling efficiency for the two proteins. It was suggested that the increased coupling reactivity results from a salt-induced association between the protein and the surface of the affinity support thereby increasing the effective concentration of protein near the epoxide reactive sites. The variation in coupling observed for different proteins at a given concentration of salt could result from differences in the susceptibilities of individual proteins to salt-induced partitioning along the surface of the support, combined with variations due to differences in the inherent reactivities of the proteins.

A close analogy has been drawn between the salt-induced solute–stationary phase interactions responsible for retention in hydrophobic interaction chromatography (HIC) and solute–solute interactions associated with precipitation in the salting-out effect. An underlying explanation for these phenomena and their close similarities has been reported [2–5]. This theory describes salt induced interfacial interactions as representing a balance between electrostatic contributions, which tend to support solvation of the solute, and hydrophobic interactions which favor solute–solute or solute–stationary phase interac-

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0021-9673/93/$06.00

tions. The theory may be extended to describe the salt induced protein–affinity phase complex formation postulated above.

Other investigators [6,7] have used conditions at high salt concentrations for the efficient coupling of proteins to silica-based epoxide affinity phases with the retention of biological activity as evidenced by the affinities of the bound moieties for mobile phase components. Dependence of protein immobilization on salt concentration has also been described for coupling to polymer-based epoxide and azyl-lactone activated supports [5,8,9]. In this investigation the extent of coupling for a collection of proteins was measured on an epoxide affinity support over a wide range of salt concentrations. Using a general first-order kinetics model to describe the relationship between soluble and immobilized protein, an expression was derived for this reaction.

EXPERIMENTAL

Materials

The proteins used in this study were obtained from Sigma (St. Louis, MO, USA). The salts and reagents used for pH adjustments are products of J.T. Baker (VWR, Brisbane, CA, USA) and were reagent grade or better. The epoxy activated affinity phase, Hydropore-EP (12 mm, 300 Å), is a product of Rainin Instrument Company (Woburn, MA, USA).

Immobilization of proteins on the affinity support

Protein immobilization was performed as previously described [1]. The protein was dissolved in 0.010 *M* potassium phosphate, pH 7.0, and to this solution was added varying amounts of 3.0 *M* ammonium sulfate in 0.010 *M* potassium phosphate, pH 7.0, resulting in protein solutions containing increasing concentrations of salt. Typically, 6 to 8 concentrations were examined for each protein, ranging at regular intervals from 0 to 1.0–2.8 *M* ammonium sulfate, depending on the protein. A 500-μl volume of the above solutions was then combined with 50 mg of the affinity support in a 1.5-ml capped vial. Each sample contained 500 μg of protein with the exception of avidin and IgG_1k monoclonal antibody which contained 400 and 167 μg, respectively. In all cases the protein solution was observed to ensure that the solubility had not been exceeded at a particular ammonium sulfate concentration.

The mixture was turned overnight by orbital rotation at room temperature and at an angle and speed which effected thorough mixing. This was achieved in the following way. A common 500-ml polyethylene bottle was attached to an overhead stirring motor, co-axial with the stirring shaft. The vials containing the coupling mixtures were attached to the surface of the bottle, vertical with respect to the bottle and motor shaft. The motor was then tilted about 45° from the horizontal. The result was that as the motor turned the bottle, the vials were rotated such that the contents turned continuously over the course of the reaction. Best mixing was achieved at low motor speeds. The reactions were carried out for 20 h except in the case of lysozyme and albumin which reacted for 25 h. The derivatized supports were then centrifuged, twice washed in 500 μl of 0.010 *M* potassium phosphate, pH 7.0, and in 1000 μl of 0.10 *M* sodium acetate, pH 4.5, containing 0.3 *M* sodium chloride. The supernatant from the washes was combined with that recovered following the reaction.

The amount of protein bound to the support in the immobilization was calculated from the difference between the amount of protein initially added and that recovered in the post-coupling wash. This was deduced from the spectrophotometric absorbance (Hitachi Model U-2000, San Jose, CA, USA) at 230 nm, except in the cases of α-chymotrypsinogen A, α-chymotrypsin, myoglobin and ribonuclease A which were measured at 280 nm. For these calculations it was assumed that variations in the extinction coefficient with salt concentration were small for the range of concentrations in the study. Most measurements were made at final salt concentrations (after dilution in the wash step) of less than 0.5 *M*, with a few measurements made at up to 0.7 *M*. Also, in nearly all cases the data set for each protein was obtained in one sitting. This eliminates the possibility of error within a data set arising from inconsisten-

cies in the wavelength setting. Determined on separate days were the two data points corresponding to the highest concentration of salt in the measurements for ribonuclease A, avidin and lysozyme.

RESULTS AND DISCUSSION

In this study we examined the efficiency with which proteins react with an epoxide affinity support in the presence of increasing concentrations of ammonium sulfate. Thirteen proteins were studied and the results obtained for percent immobilization as a function of ammonium sulfate concentration are shown in Figs. 1 and 2. Table I shows the approximate concentration of ammonium sulfate required for 90% immobilization in 20 h. All but three of the proteins exhibited 95–100% immobilization within 20 h, and the salt concentration at which this occurred depended on the individual protein examined. Of those that failed to react completely, ribonuclease A attained 85–90% immobilization while α-chymotrypsinogen A reached 60–65% and lysozyme attained only about 50% immobilization.

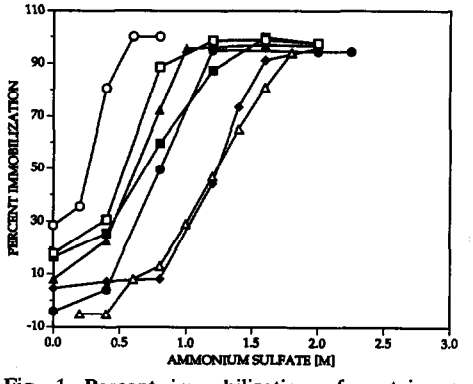

Fig. 1. Percent immobilization of proteins on an epoxy affinity support *vs.* ammonium sulfate concentration for proteins exhibiting elevated immobilization at zero salt concentration. Protein was dissolved in solutions containing varying amounts of ammonium sulfate. The protein solution was reacted with the epoxy affinity phase and after reaction the remaining protein was removed from the particles by washing. The amount of protein bound to the support was determined spectrophotometrically by the difference between the amount of protein added and the amount of protein in the washes. See Experimental for details. ○ = Conalbumin; ▲ = α-chymotrypsin; ● = α-chymotrypsinogen A; □ = ribonuclease A; ◆ = lysozyme; ■ = avidin.

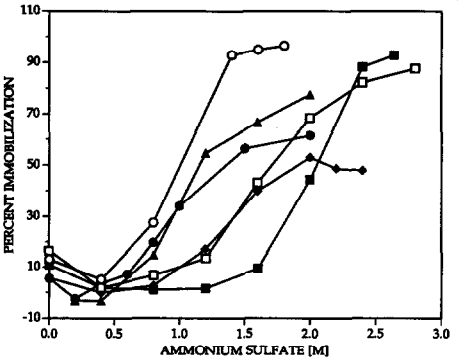

Fig. 2. Percent immobilization of proteins on an epoxy affinity support *vs.* ammonium sulfate concentration for proteins which did not exhibit elevated immobilization at zero salt concentration. See Fig. 1 for details. ○ = IgG_1k; □ = transferrin; ▲ = α-lactalbumin; ■ = myoglobin; ● = bovine serum albumin; △ = carbonic anhydrase; ◆ = β-lactoglobulin B.

The strong dependence of the degree of protein immobilization on the affinity support with respect to ammonium sulfate concentration led us to examine the fraction of immobilization of protein as a function of salt concentration. Although all the proteins exhibited increases in immobilization beginning at some concentration of salt, the concentration thresholds at which this was observed varied widely among the proteins examined. For some proteins, such as IgG, transferrin and β-lactoglobulin B, shown in Fig. 1, increases in immobilization were first observed at salt concentrations below 0.5 *M*. Others, such as avidin and ribonuclease A, shown in Fig. 2, required concentrations above 1.0 *M* to exhibit significant increases.

The reaction between proteins and the epoxide ligands on the affinity support can be represented by the reaction scheme

$$P + L \underset{k_{-1}}{\overset{k_{+1}}{\rightleftharpoons}} P \cdot L \xrightarrow{k_2} P - L$$

where P is free protein in solution, L is the stationary phase containing the epoxy groups, $P \cdot L$ is the noncovalently bound protein on the stationary phase, and P–L is the protein covalently bound to the surface through reaction with the epoxy groups. According to this model, any perturbation in the immobilization mixture

TABLE I

AMMONIUM SULFATE CONCENTRATION REQUIRED FOR 90% IMMOBILIZATION OF PROTEINS ON HYDROPORE-EP EPOXIDE AFFINITY SUPPORT

Protein	Molecular weight	p*I*	Ammonium sulfate concentration (*M*)
Lysozyme[a]	14 000	11.0	–
Avidin	68 000	10–10.5	2.4
α-Chymotrypsinogen A[a]	25 000	9.5	–
Carbonic anhydrase[b]	29 000	5.4, 5.9, 6.6	1.7
Ribonuclease A[c]	13 700	9.4	2.8 (87%)
α-Chymotrypsin[c]	21 600	8.8	2.0 (77%)
Myoglobin	17 500	7.1	1.3
IgG_1k monoclonal antibody	55 000	NA	0.4
Conalbumin	77 000	6.5	1.4
Transferrin	77 000	5–6	0.8
β-Lactoglobulin B	35 000	5.1	1.6
Serum albumin (bovine)	68 000	4.4–4.8	1.1
α-Lactalbumin	14 200	4.5–4.7	0.9

[a] These proteins were not 90% immobilized. See text.
[b] The isoelectric points shown represent three isozymes, all or part of which may have been present.
[c] This was the highest concentration examined.
NA = Not available.

which shifts the equilibrium to increase the ratio of $[P \cdot L]/[P][L]$ will increase the concentration of protein near the epoxide groups along the surface of the support. An increase in the concentration of $P \cdot L$ will result in a faster rate in the formation of the product $P - L$.

When the reaction is stopped after 20 h the amount of protein (P_m) measured in solution is the sum of P and $P \cdot L$. The rate constant (k_2) for the attack of a protein associated nucleophile on an epoxy group is assumed to be small compared to both the rate constant of formation (k_{+1}) and the rate constant (k_{-1}) for breakdown of $P \cdot L$.

Based on this assumption the concentration of $P \cdot L$ can be represented as

$$[P \cdot L] = [P][L]K \quad (1)$$

where $K = k_{+1}/k_{-1}$.

Then

$$d[P - L]/dt = k_2[P \cdot L] = k_2K[L][P]$$
$$= k_2K[L_0][P] \quad (2)$$

where $[L_0]$ is the initial concentration of epoxide ligands on the stationary phase and is large compared to $[P \cdot L] + [P - L]$. From the identities $[P_0] - [P_m] = [P - L]$ and $[P_m] = [P]\ (1 + K[L_0])$ eqn. 2 can be modified and integrated to yield

$$\log f = -k_2K[L_0]t/(2.3)(1 + K[L_0]) \quad (3)$$

where f is the fraction of protein which is not covalently bound to the stationary phase and is equal to $[Pm]/[P_0]$. In these experiments f was determined after a constant time (20 h). A plot of log f_a (f_a is the fraction of free protein remaining in solution after 20 h) as a function of ammonium sulfate concentration for ribonuclease A, lysozyme and α-chymotrypsinogen A is shown in Fig. 3.

At ammonium sulfate concentrations where K is small eqn. 3 reduces to

$$\log f_a = -k_2K[L_0]t_a/2.3 \quad (4)$$

where t_a is the duration of the reaction (20 h). According to eqn. 4 log f_a is directly proportional to K. Partition of the protein along the epoxide phase, represented by the equilibrium constant K in eqn. 4, is assumed to be a function

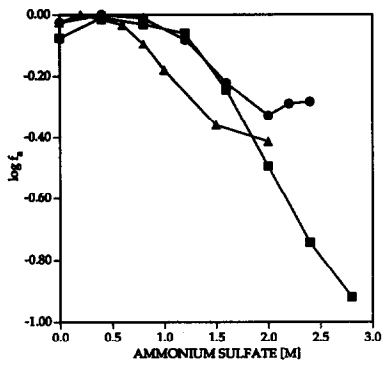

Fig. 3. Log f_a *vs.* ammonium sulfate concentration. See Fig. 1 for details. ■ = Ribonuclease A; ● = lysozyme; ▲ = α-chymotrypsinogen.

of salt concentration, analogous to the salt induced associations which occur between proteins and the stationary phase in hydrophobic interaction chromatography (HIC). Horváth and coworkers [2,3] have shown that for HIC the distribution of the protein solute between the stationary and mobile phases parallels the dependence of protein solubility on salt concentration. Protein solubility at very low salt concentrations at first increases with increasing salt concentration (the salting-in effect), and then decreases as the salt approaches high concentration (the salting-out effect). The logarithmic dependence of protein solubility is non-linear with respect to salt at low concentrations and becomes linear at higher concentrations, a characteristic which is also found for log k' *vs.* salt concentration in HIC [2]. In this study trends similar to these were found for the salt dependence of protein immobilization on the epoxide support. The dependence of log f_a (or percent immobilization) on ammonium sulfate concentration appears to be non-linear at low salt concentrations and approaches a linear dependence at higher concentrations. Plots of log f_a *vs.* ammonium sulfate concentration are shown in Fig. 3 for three proteins.

Some of the proteins in this study, seen in Fig. 1, also exhibited a slight increase in immobilization (lowered f_a) as the concentration of ammonium sulfate approached zero. Such an increase in immobilization could occur, according to the above argument, as a result of an enhanced association between the protein and support in the absence of salt. Protein–stationary phase associations like these have been reported [10] for the isocratic retention of proteins on a silica-based HIC stationary phase (polyvinyl alcohol). In that study the retention of some proteins first declined as ammonium sulfate concentration increased just above zero concentration. Although increases in HIC protein retention in the absence of salt or the increases in immobilization seen in this study are reminiscent of the salting-in effect, another explanation is possible. The proteins which demonstrated this characteristic were all basic (except conalbumin, p*I* 6.5) and should possess a net positive charge at pH 7 while the epoxy phase would be expected to have a net negative charge due to any unreacted silanols present on the underlying surface of the silica. In the absence of ammonium sulfate these proteins would show enhanced association with the solid phase through ionic interactions which would lead to relative increases in immobilization. In the HIC study described above the proteins which exhibited low salt associations with the stationary phase were also basic proteins. It can also be noted that the proteins in this study which did not show elevated immobilization at zero salt concentration all possess isoelectric points near or below p*I* 7.0. These proteins are shown in Fig. 2.

When K becomes large eqn. 3 reduces to

$$\log f_a = -k_2 t_a / 2.3 \quad (5)$$

which defines a region of salt concentrations where the fraction of immobilized protein is independent of K. In this region of salt concentrations a large fraction of the protein is already associated with the solid phase and any increase in salt concentration would not lead to an appreciable increase in P·L. The extent of immobilization in this region of salt concentrations is only a function of the rate constant k_2. For most proteins studied (*e.g.*, ribonuclease A in Fig. 3) the rate constant k_2 is large enough that complete immobilization occurs within 20 h under the conditions described by eqn. 5. For two proteins, lysozyme and α-chymotrypsinogen A, k_2 is small, and less than complete immobili-

zation occurs in 20 h even though all the protein is associated with the stationary phase (Fig. 3).

These results clearly indicate that relatively high concentrations of ammonium sulfate are required for satisfactory immobilization of proteins on the epoxide support, and that, in most cases, very high coupling efficiencies can be achieved if this condition is met. The ability to achieve high coupling efficiencies at a neutral pH allows immobilization to be performed in the absence of pH extremes and without the addition of potentially denaturing reagents, thereby helping to preserve the integrity of the immobilized ligand in the coupling process.

ACKNOWLEDGEMENTS

This work was supported by Rainin Instrument Company, Inc.

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