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Salt-induced immobilization of small affinity ligands on an epoxide-activated affinity support

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Abstract

The salt-induced immobilization of small ligands onto an epoxy-activated HPLC stationary phase was found to be an effective route to the preparation of HPLC-compatible affinity supports. The ligands S-methylglutathione, S-butylglutathione and S-octylglutathione were immobilized at pH 10.5 under various conditions of salt type and concentration. In potassium carbonate buffer the extent of reaction for the alkylglutathione ligands was insensitive to carbonate concentration (0.5–2 *M*) but was substantially influenced by the hydrophobicity of the ligand. S-Octylglutathione was immobilized with a 4-fold greater efficiency in 2 *M* potassium carbonate than was the less hydrophobic S-methylglutathione. By contrast, in potassium phosphate buffer the extent of the immobilization was strongly dependent on salt concentration. For the S-alkylglutathiones, a 2-fold increase in immobilization efficiency was found when increasing the phosphate concentration from 0.5 to 2 *M*. Prior theory was extended to explain these results involving a salt-induced hydrophobic interaction between the ligand and the epoxy phase which in turn results in faster reaction rates between ligand and epoxy groups. As predicted by this model, the immobilization of the very hydrophilic ligand, nitrilotriacetic acid, was found to be affected minimally by either salt type or salt concentration. Further, when the rate of hydrolysis of the immobilized epoxy groups was studied, the maximum amount of immobilized ligand was found, in some cases, to be limited by the hydrolysis of the epoxy groups. Practical application of these principles allowed a nickel chelate HPLC-affinity phase to be produced using the nitrilotriacetic acid as the ligand. The HPLC phase was found to be useful in multiple, small-scale purifications of a histidine-tagged protein kinase C fragment.

Keywords: Affinity absorbents; Alkylglutathione ligands; Nitrilotriacetic acid ligand; Immobilization, salt-induced; Epoxy-activated stationary phases; Stationary phases, LC; Protein

1. Introduction

Epoxy-activated chromatographic supports have been widely used for many applications in affinity separations. Attachment of very diverse ligands, such as proteins [1,2], nucleic acids [3] and various small molecules [4], to the support via nucleophilic addition to the epoxide ring have been successfully applied. Although epoxy-activated phases have been criticized for being unreactive compared to other activated phases [5], a technique has been described for overcoming this problem [1,2]. In the presence of

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high concentrations of certain salts, efficient immobilization of proteins at pH 7 has been reported. High salt concentrations were also used to increase the efficiency of immobilization of oligonucleotides to an epoxy-activated phase at neutral pH [3]. The increased coupling reactivity was explained as resulting from a salt-induced association between the macromolecule and the surface of the affinity support, thereby increasing the effective concentration of the nucleophilic groups on the macromolecule near the epoxide reactive sites [1–3]. This increase in effective concentration leads to an increase in reaction rate leading to efficient immobilization. The increased association between macromolecule and the stationary phase is the result of an increase in hydrophobic interactions at high salt concentrations as described for hydrophobic interaction chromatography and for the general salting-out effect [6,7].

The immobilization of small molecules on epoxy-activated phases (Fig. 1A) is generally carried out at higher pH values, from 9 to 11, so that the reactivity of free amino groups can be utilized [4]. These pH conditions can be used since small molecules are often not as susceptible to degradation at high pH as are macromolecules. Attachment of the small molecule affinity ligand via nucleophilic addition of an amino group to the epoxy moiety has a potential advantage for affinity chromatography at physiological pH, because the resulting amino group in the coupled product is able to retain its positive charge. Since the binding of small molecules to a protein site often has an electrostatic component, retention of this positive charge is probably important. For many affinity activation chemistries, the amino group on the affinity ligand becomes attached to the stationary phase via the formation of an amide bond. At physiological pH, this nitrogen in the amide bond is uncharged and may result in decreased binding between the affinity ligand and the protein site.

Often small molecule affinity ligands are not available in large quantities and a technique to efficiently couple these to the epoxy phase is of value. In the present investigation the effect of salt concentration and salt type is studied for the immobilization at pH 10.5 of three tripeptides of increasing hydrophobicity and of a highly polar ligand, nitrilotriacetic acid (NTA). In addition, the hydrolysis of the epoxy groups (Fig. 1B) is measured to determine whether the availability of epoxy groups is limiting the extent of small ligand immobilization. The NTA phase is further used as a nickel affinity phase to purify a hexahistidine-tagged recombinant protein fragment of θ PKC.

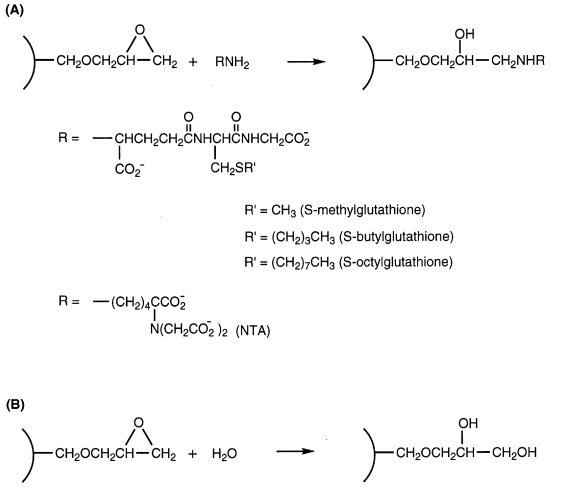

Fig. 1. Reaction of epoxy-activated affinity phase. (A) Small molecule ligand, and (B) hydrolysis by water.

2. Experimental

2.1. Reagents

Iodobutane, iodooctane, the sodium salt of mercaptoacetic acid, disodium EDTA, nickel sulfate hexahydrate and 1,4-butanediol diglycidyl ether were purchased from Aldrich (Milwaukee, WI, USA). Glutathione, S-methylglutathione, and Tris (Sigma-Ultra) were obtained from Sigma (St. Louis, MO, USA). Dabsyl chloride was obtained from Pierce (Rockford, IL, USA). HEMA BIO 1000 (10 and 20 μm) was purchased from Melcor Technologies (Sunnyvale, CA, USA). Other reagents were of analytical grade.

2.2. Synthesis of peptides

S-Butylglutathione and S-octylglutathione were synthesized by the method of Vince et al. [8]. All peptides had greater than 90% purity when analyzed

by reversed-phase HPLC and had acceptable elemental analyses.

2.3. Synthesis of N-(5-amino-1-carboxypentyl)iminodiacetic acid (NTA)

The NTA was synthesized by the method of Hochuli et al. [9] with the following changes. Before hydrogenation, *N*-(5-benzyloxycarbonylamino-1-carboxypentyl)iminodiacetic acid was carefully dissolved in water by adding the appropriate amount of 10 M potassium hydroxide to a final pH of 7. After hydrogenation no attempt was made to isolate the resulting NTA because of the deliquescent nature of this material. The hydrogenation was assumed to proceed to completion, and the volume was reduced by approximately 80% under reduced pressure. This reduction in volume removed any residual toluene. Analysis of the resulting solution showed only one product spot by TLC. The concentration of NTA was accordingly calculated based on the original amount of *N*-(5-benzyloxycarbonlyamino-1-carboxypentyl)-iminodiacetic acid.

2.4. Chromatography

The HPLC system consisted of two HPXL pumps equipped with 10 ml/min titanium pump heads, a Rheodyne 7125-081 titanium injector, Dynamax software and a Dynamax UV-C detector (Rainin Instrument Co., Woburn, MA, USA). An intensity of 1000 mV was equivalent to 1 O.D. unit. All pathways in contact with the mobile phase were of either titanium or biocompatible polymer construction.

For affinity chromatography, a gradient was formed with a static mixing tee (Upchurch Scientific, Oak Harbor, WA, USA). Biocompatible 250 p.s.i. back-pressure regulators (Upchurch Scientific) were placed between the injector and the mixing tee. The back-pressure regulators were placed in-line to insure proper function of the HPLC pump check valves at the low pressure generated at low flow-rates. A six-port low-pressure selection valve of polyether ether ketone (PEEK) construction mounted on an electric actuator was obtained from Upchurch Scientific. The low-pressure selection valve was controlled by Dynamax software and was used in the equilibration of the nickel-loaded affinity column during the isolation of the PKC θV1 fragment (see legend to Fig. 3).

2.5. Affinity matrices

The synthesis of the base epoxy material follows the general procedure of Sundberg and Porath [10]. HEMA BIO 1000 (700 mg), 1,4-butanediol diglycidyl ether (2.3 ml) and 0.5 *M* sodium hydroxide (2.3 ml) were combined in a 13×100 mm culture tube equipped with a PTFE-lined screw cap (VWR Scientific Products, Brisbane, CA, USA), sonicated for 5 min, and then mixed by rotation for 17 h. It was found that this reaction could be scaled-up by a factor of three using a 20×125 mm culture tube equipped with a PTFE-lined screw cap (Aldrich, Milwaukee, WI, USA). The particles were filtered and washed with at least 10 bed volumes each of water, ethanol and acetone.

For the S-alkylglutathione phases, 200 mg of the dried epoxy matrix weighed into 2-ml vials with PTFE enclosures were mixed with 1 ml of solutions adjusted to pH 10.5 of S-octylglutathione (40 mg/ml), of S-butylglutathione (34 mg/ml), or of S-methylglutathione (30 mg/ml), dissolved in the appropriate buffer listed in Table 1. Following sonication for 5 min, the suspensions were mixed by rotation at 23° for 90 h. After filtration the particles were washed with at least 10 bed volumes each of the following solutions: (i) 1 *M* sodium chloride, 0.1 *M* sodium phosphate (pH 9), (ii) 1 *M* sodium chloride, 0.1 *M* sodium acetate (pH 4.5), (iii) water, (iv) ethyl alcohol and (v) acetone.

For the NTA phases, 200 mg of the dried epoxy matrix weighed into 2-ml vials with PTFE enclosures were mixed with 1 ml solutions adjusted to pH 10.5 of NTA dissolved in the appropriate buffer listed in Table 1. Following sonication for 5 min, the suspensions were rotated for 90 h at the indicated temperature. After filtration the particles were washed with at least 10 bed volumes each of the following solutions: (i) 1 *M* sodium chloride, 0.1 *M* sodium phosphate (pH 9), (ii) 1 *M* sodium chloride, 0.1 *M* sodium acetate (pH 4.5), (iii) water, (iv) ethyl alcohol and (v) acetone.

Table 1
Immobilization of NTA and S-alkylgluthathiones as a function of salt type and salt concentration

Buffer	NTA[a]		S-Methylglutathione[a]		S-Butylglutathione[a]		S-Octylglutathione[a]	
	μmol/g	% Immob.	μmol/g	% Immob.	μmol/g	% Immob.	μmol/g	% Immob.
2.0 *M* Carbonate	29.0±1. 0	2	16.1±0. 6	3	40.9±0. 5	9	68.5±1. 4	15
1.0 *M* Carbonate	24.9±0. 1	2	21.3±2. 0	4	35.4±2. 1	8	67.6±1. 3	14
0.5 *M* Carbonate	25.8±0. 5	2	29.0±0. 7	6	40.3±0. 4	9	67.7±0. 6	14
2.0 *M* Phosphate	32.9±3. 5	2	83.8±2. 8	18	111.1±4.0	24	92.9±2. 4	20
1.0 *M* Phosphate	34.4±2. 8	2	56.2±4. 1	12	91.8±14.6	20	84.3±2. 2	18
0.5 *M* Phosphate	35.9±1. 2	2	32.7±0. 6	7	48.5±9. 1	10	57.0±2. 2	12

[a]Per gram of epoxy-activated packing, the amounts of ligand initially in solution (μmol) were 1530 for NTA, 470 for methyl glutatione, 470 for butyl glutathione, and 470 for octyl glutathione.

2.6. Packing of columns

The affinity material (650 mg) was slurried in 20 ml of water and was packed at high pressure into stainless steel columns 0.46×3 cm (Supelco, Bellefonte, PA, USA). The column frits were 2 μm (average pore diameter) titanium encased in a CTFE ring (Upchurch Scientific). A Haskell (Burbank, CA, USA) DSTV122 liquid pump was used to provide the drive solvent (water) during the packing process. The columns were packed at 2000 p.s.i. with 50 ml of water and then 4000 p.s.i. with a further 50 ml of methanol (1 p.s.i.=6894.76 Pa). The two-stage column packing procedure was adopted after a packing study. This method gave good peak shape for the lifetime of the column.

2.7. Analysis of the epoxy content of the base affinity phase

Based on the method of Scoble and Scopes [11], the epoxy-activated phase was reacted with mercaptoacetic acid and the number of acidic groups introduced was determined by titration. A stock solution (pH 9.5) of 1 *M* sodium mercaptoacetate in 2 *M* sodium carbonate was prepared immediately before each analysis and was saturated with argon. Six ml of the mercaptoacetate solution was then added to approximately 150 mg of the epoxy phase in a 13×100 mm culture tube equipped with a PTFE-lined screw cap. The space above the suspension was swept with argon, the tube was sealed, and the suspension was sonicated for 5 min. The suspension was either rotated at 23°C or heated at 60°C in a Reacti-Therm III Heating Module (Pierce). After reaction, the material was filtered and washed with at least 10 bed volumes of water followed by 0.1 *M* hydrochloric acid. The resulting cake was then suspended in 8 ml of 0.1 *M* hydrochloric acid, sonicated for 5 min, and filtered again. The resulting material was washed with at least 10 bed volumes of water followed by 10 bed volumes of acetone. After air drying, approximately 140 mg of this material was precisely weighed into a 4-ml vial containing a magnetic stir bar. Two milliliters of 0.1 *M* sodium chloride was added to the particles, the suspension was sonicated for 5 min, and the stirred mixture was titrated with 0.100 *M* Tris added portionwise. After each addition of Tris, the pH was recorded using a pH meter. The pH was plotted as a function of the amount of Tris added and the end-point of the titration determined from the inflection point of the plot which occurred at pH 6.4 [11]. Analyses were performed in triplicate and the range in data was less than 3%.

2.8. Analysis of the S-alkylglutathione phases

The analysis of peptides on supports was accomplished by a modification of the method reported by Knecht and Chang [12]. Two to 8 mg of dried support was weighed into the hydrolysis tubes. S-Methylglutathione, S-butylglutathione, and S-octylglutathione peptides were used as standards. These peptides were dissolved at a concentration of 10 μmol/ml in a solution of 0.05 *M* sodium hydroxide (ethanol–water, 25:75%). Isoleucine dissolved in water at a concentration of 10 μmol/ml was used as an internal standard. Hydrolysis tubes containing 5–50 μl of the standard peptide solutions were

evaporated to dryness in a Sorval Speed Vac. Twenty-five microliters of the isoleucine standard were added to all tubes containing samples and peptide standards followed by the addition of 500 μl of 6 *M* hydrochloric acid. The tubes were capped, and placed in a heat block at 138°C for 18 h. Six concentration levels of each peptide standard were used from 50 to 500 n*M*.

The tubes containing the acid hydrolysates were vortexed, centrifuged, and 50 μl of the hydrolysate was added to a 1.5-ml polypropylene centrifuge tube. The acid was evaporated off under vacuum. Then 150 μl of 0.1 *M* sodium carbonate buffer (pH 9.0), and 150 μl of 4 nmol/μl dabsyl chloride solution in acetonitrile were added and vortexed. With the tubes tightly capped, the mixture was heated for 10 min at 75°C, vortexed, and heated for another 5 min at 75°C. After dabsylation, the samples were diluted by the addition of 300 μl of 0.05 *M* sodium phosphate (pH 6.8)–ethanol (1:1, v/v), vortexed, and centrifuged at 13 000 r.p.m. for 15 min. Twenty microliters of the solution were injected onto the HPLC.

The dabsylated amino acids were separated on a Supelcosil LC-18, 150×4.6 mm column (Supelco). Mobile phases were: (A) 95% 0.01 *M* sodium phosphate (pH 7.0), 5% acetonitrile; (B) 30% 0.01 *M* sodium phosphate (pH 7.0), 70% acetonitrile. The gradient used was 20% B to 51% B in 17.2 min, 51% B to 81% B in 3.5 min, linear at 81% B for 2.5 min, 81% B to 100% B in 1 min, linear at 100% B for 2 min, and then back to 20% B in 1 min. The flow-rate was 1.4 ml/min. The detector wavelength was 436 nm. All HPLC analyses were performed at ambient temperature. The amino acid concentration was calculated from the six-level standard concentration curve using the peak height ratio of each amino acid to the internal standard. Analyses were performed in triplicate and the range in data is indicated by the error.

2.9. Analysis of the NTA phases

Approximately 60 mg of a NTA phase was weighed into a 4-ml vial with PTFE enclosures and 3 ml of a 200 m*M* nickel sulfate solution was added. The suspension was sonicated for 5 min and then mixed by rotation for at least 12 h. The nickel phase was filtered and washed with at least 10 bed volumes each of (i) water, (ii) 0.2 *M* acetic acid, 0.2 *M* sodium chloride, (iii) water, (iv) ethanol and (v) acetone. After air drying, approximately 40–60 mg of nickel-loaded phase was precisely weighed into a 2-ml vial with PTFE enclosures and 1.5 ml of 0.1 *M* EDTA was added. The resulting suspension was sonicated for 5 min and rotated for 12 h. After centrifugation, approximately 1 ml of supernatant was carefully removed and this solution was analyzed by atomic adsorption spectroscopy. Analyses were performed in triplicate and the range in data is indicated by the error range.

2.10. Expression of PKC θV1

A plasmid was designed to direct the prokaryotic expression of the θPKC variable domain containing an N-terminal hexahistidine-tag for one-step purification and a C-terminal c-myc epitope tag for immunological detection. The prokaryotic expression vector, pQE-30 (Qiagen, Chatsworth, CA, USA), was modified by restriction endonuclease digestion with *Sph*I and *Hin*dIII (which removes most of the polylinker region), followed by insertion of an oligonucleotide cassette. This cassette maintains the 5′ His-tag coding region of pQE-30, regenerates the unique *Sph*I site, and introduces a unique *Not*I site for cDNA cloning. The sequence also encodes an in frame c-myc epitope tag (AEQKLISEEDLN) downstream from the *Not*I site for immunological detection of recombinant protein. Oligos were first annealed and ligated into the restricted plasmid and then transformed into the bacterial strain XL1-Blue (Stratagene, La Jolla, CA, USA) by standard procedures. The unique *Sph*I and *Not*I cloning sites were used for insertion of PCR-generated cDNAs. The new vector generated, pHISTER-3, was used for the expression of the human PKC θV1 variable domain in *E. coli*.

Oligo dT-primed RT-cDNA from the human Jurkat T-cell leukemic line was used for amplification of variable domain 1 of the human PKC θ isozyme (corresponding to amino acids 1–140). Primary amplifications were performed using oligos which introduce 5′ *Sph*I and 3′ *Not*I restriction sites, respectively. The reactions contained 25 pmol each of: appropriate primer, Perkin-Elmer *Taq* polymerase buffer with $MgCl_2$ added to 2 m*M*, 5 μl of RT-

cDNA reaction mix, all four dNTPs at 250 μ*M* each, and 2.5 units of *Taq* polymerase in a final volume of 50 μl. Using an Omnigene thermal cycler, the reaction mixtures were put through 25 cycles of denaturation (94°C, 1 min), annealing (60°C, 1 min), and extension (72°C, 1 min) followed by a final cycle of extension (72°C, 10 min). The reaction products were gel purified, digested with *Sph*I and *Not*I, repurified on agarose gels and ligated into *Sph*I/*Not*I cut pHISTER-3 vector by standard procedures. Ligated plasmids were transformed into *E. coli* strain XL1-Blue using a protocol described by the manufacturers (Stratagene). All plasmid constructs were verified by sequencing on an ABS 373 automated DNA sequencer (Applied Biosystems, Foster City, CA, USA).

Recombinants in the XL1-Blue bacterial strain were used for expression of recombinant proteins. Overnight cultures containing the appropriate plasmid constructs were grown at 37°C in LB containing 100 μg/ml ampicillin and 1% glucose. These cultures were diluted 1:100 in LB containing 100 μg/ml ampicillin and 0.1% glucose and incubated at 37°C with shaking until the $O.D._{600}$ was 0.6–0.8, after which time IPTG was added to a final concentration of 1 m*M*. The cultures were then incubated at 30°C with shaking for 5 h. The cells were harvested by centrifugation at 6600 r.p.m. in a Sorval SS-34 rotor for 20 min at 4°C. Cell pellets were resuspended in 10 volumes of lysis buffer (50 m*M* sodium phosphate, pH 8.0, 300 m*M* sodium chloride), frozen and thawed on dry ice–ethanol and sonicated on ice 4×15 pulses with a Branson sonifier at 50% duty cycle and output setting 6. The lysate was centrifuged at 16 000 r.p.m. in a Sorvall SS-34 rotor for 20 min at 4°C.

3. Results

The number of epoxy groups on the hydroxyethyl methacrylate (HEMA) resin used in this study was determined using the method of Scoble and Scopes [11]. In this method epoxy groups are reacted with mercaptoacetic acid at elevated pH, the resin is washed with hydrochloric acid and the resulting immobilized acid groups are titrated with 0.1 *M* Tris solution. Although it is not possible to demonstrate that a reagent reacts to completion with the epoxy groups, for the purposes of this assay it has been deemed sufficient to find conditions where maximum reaction occurred [11]. For the epoxy-activated HEMA resin, a condition for maximum reaction at pH 9.5 was found to be 1 *M* mercaptoacetic acid for 16 h at 60°C. At 60°C, analysis of acidic groups after 16 and 65 h showed the same extent (100%) of reaction had occurred. At pH 10.5, an equal number of titratable groups was found to those found when the reaction was conducted at pH 9.5. At pH 9.5, 82% of the epoxy groups were reacted in 12 h at room temperature.

The number of acidic groups on the HEMA resin that were not attributable to immobilization of mercaptoacetic acid via epoxy groups was determined in two ways. First the HEMA was treated as described in the Section 2 for the activation of the resin, except that 1,4-butanediol diglycidyl ether was not included and the volume of 0.5 *M* sodium hydroxide was doubled. The resulting material was then treated with mercaptoacetic acid at 60°C as described in Section 2, and the number of acidic groups determined. The number of titratable groups was found to be 23 μmol/g. In a second experiment, epoxy-activated HEMA was exposed at room temperature to mercaptoacetic acid, the mixture immediately filtered and the resulting material was washed as described in Section 2. The number of titratable groups found under these conditions was found to be 21 μmol/g. In all calculations to determine the number of epoxy groups on the activated resin, a background concentration of 22 μmol/g of acidic groups was subtracted from the number of acidic groups determined by the mercaptoacetic acid method. For three separately synthesized batches of the epoxy-activated HEMA, the number of epoxy groups determined by this method was 138, 151 and 158 μmol/g. To determine the rate of hydrolysis of the epoxy groups (Fig. 1B), 2 g of epoxy-activated HEMA was stirred with 8 ml of 2 *M* potassium carbonate solution (pH 10.5). Two portions of 0.6 ml were removed at 0, 24, 48, 72, 96, 120 and 198 h and analyzed for epoxy content. The results are shown in Fig. 2.

The effect of salt type and salt concentration on the extent of reaction of NTA and S-alkylglutathiones with the epoxy-activated HEMA

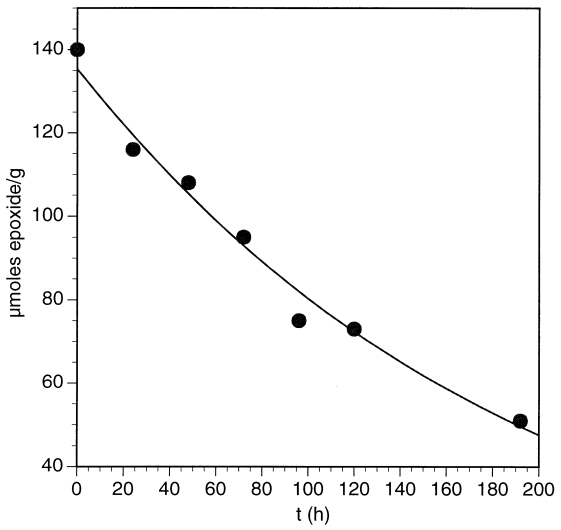

Fig. 2. Rate of hydrolysis of epoxy-activated HEMA at pH 10.5.

was investigated at 23°C and pH 10.5. The appropriate concentration of potassium carbonate or potassium phosphate was used to dissolve the NTA or S-alkylglutathione and the pH of the resulting solution was adjusted to 10.5. After checking visually to see that no precipitate had formed, the solution was added to the epoxy-activated HEMA and allowed to react for 90 h. The resulting mixtures were filtered and submitted for analysis to find the amount of bound peptide in the case of the S-alkylglutathiones or nickel in the case of NTA. It was assumed that the amount of NTA immobilized on the resin was equivalent to the amount of nickel determined. The results are shown in Table 1. For all the alkyl glutathione and NTA phases, the same batch of epoxy-activated HEMA was used.

The percentage of immobilized ligand is determined from the ratio of the amount of ligand bound per gram of resin after 90 h to the amount of ligand per gram of resin in solution at the beginning of the reaction. For the reaction of NTA with the epoxy-activated HEMA, several temperatures were investigated. At 23 and 37°C, equivalent amounts of NTA were bound to the resin after 90 h at pH 10.5 while at 4°C the amount immobilized was reduced by 53%.

Using the conditions of 2 *M* potassium carbonate and pH 10.5 with a ratio of 4.7 mmol of NTA in solution per gram of resin, a packing was produced with 39 μmol/g of NTA per gram of resin and used in the purification of a recombinant protein fragment from protein kinase C θ which had six histidine residues at the N-terminus. After the resin was charged with nickel, a crude mixture containing the His-tagged fragment was injected onto the column. After washing with phosphate buffer and with a gradient from 0 to 10 m*M* imidazole, the fragment was eluted with 500 m*M* imidazole at 42 min (peak 1, Fig. 3). The column was then regenerated for the next run.

4. Discussion

The method of Scoble and Scopes [11] for the determination of epoxy groups on Sepharose CL-4B was adapted for the determination of epoxy groups on the HEMA resin. To obtain complete reaction of the epoxy groups with mercaptoacetic acid, more vigorous conditions (60°, 17 h, 1 *M* mercaptoacetic acid, pH 9.5) were required for the HEMA resin than those described for Sepharose CL-4B (room temperature, 1 h, 1 *M* mercaptoacetic acid, pH 10.5). After 2 h using their conditions, only 20% of the total epoxy groups on the activated HEMA reacted. The difference in reaction conditions probably arises because of the difference in the structure of the two resins. The HEMA resin may have a structure that limits access of mercaptoacetic acid, and hence slows the rate of reaction between the epoxy groups and thiol reagent. For the Sepharose resin, no difference was seen in the amount of mercaptoacetic acid coupled between pH 9 and 12. Likewise, for the HEMA-activated resin, no difference in the amount of mercaptoacetic acid coupled was found at 60°C whether conducted at pH 9.5 or 10.5.

The rate of hydrolysis of epoxy groups (Fig. 1B) at 23°C and pH 10.5 was measured by taking aliquots of the epoxy-activated HEMA suspended in potassium carbonate buffer (pH 10.5) at predetermined time points and determining the number of epoxy groups remaining by the mercaptoacetic acid method. The results are shown in Fig. 2 and the fitted curve is derived from the first order equation:

$$\mu\text{mol epoxy} = 135e^{-at}$$

where the first-order rate constant, $a = 0.0052\ h^{-1}$.

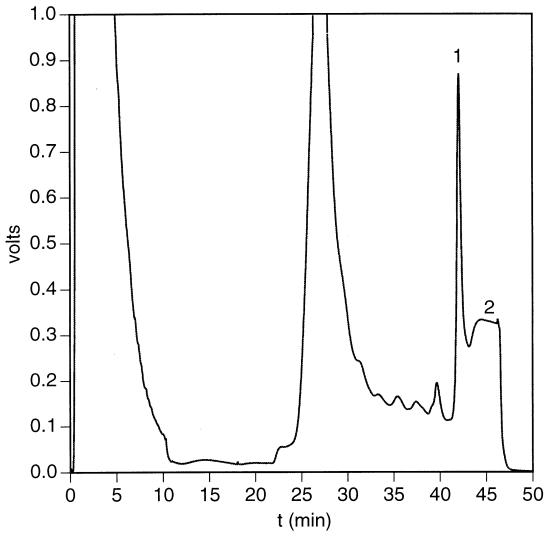

Fig. 3. Affinity chromatogram of His-tagged PKC θV1 on a nickel chelate column. Supernatant (100 μl) was loaded onto the affinity column with 200 m*M* sodium phosphate, 5% glycerol, pH 8.0 (buffer A), at 0.5 ml/min for 10 min. At 10 min the automated injection loop was switched out of the flow path and flow was increased to 4.0 ml/min and maintained for 8 min. At 18 min the flow rate was decreased to 0.4 ml/min over 1 min. At 20 min, a gradient of 0–10% 500 m*M* imidazole in buffer A was run over 20 min. At 41 min 100% 500 m*M* imidazole in buffer A was introduced and maintained for 5 min. The histidine-tagged PKC θV1 eluted and was collected within the first 2 min of the introduction of 100% 500 m*M* imidazole in buffer A. At 46 min the flow rate was increased to 4.0 ml/min in 100% 500 m*M* imidazole in buffer A for 5 min. The high flow rate was sustained during column regeneration with 5 min each of H_2O, 100 m*M* nickel sulfate in H_2O, and buffer A, respectively. At 71 min the flow rate was decreased to 0.4 ml/min to prepare the column for the next injection. Peak 1 contains the protein kinase C polypeptide fragment. Peak 2 is due to the imidazole absorbance at 280 nm.

The second-order rate constant is 2.6×10^{-8} l/mol s based on the assumption that the rate of hydrolysis at pH 10.5 is a pH-independent reaction. Previous investigation of the solution kinetics for the addition of water to epoxy groups indicates a pH-independent region from 7 to 11 [13]. The rate of water addition to the epoxy groups of activated HEMA resin is very similar to the rate of water addition in solution to the hindered isobutylene epoxide. For the hindered epoxide the second-order rate constant is 3.0×10^{-8} l/mol s [13].

The reaction of small ligands with the epoxy-activated phase was studied with S-alkyl glutathiones and NTA (Fig. 1). These small ligands were chosen because of their range of hydrophobicities and because of their usefulness in affinity chromatography. One of the S-alkylglutathiones, S-octylglutathione, has been used successfully as an affinity ligand for the enzyme class glutathione S-transferase [14,15] and NTA has been used in nickel chelate chromatography [9].

The extent of reaction of small molecules with an epoxy-activated stationary phase was generally in accord with previous findings for the extent of reaction of this type of surface with macromolecules [1,2]. As the hydrophobicity of the small molecule increases, the extent of reaction increases for a defined time period. For the S-alkylglutathiones, the hydrophobicity increases as the length of the alkyl chain increases. Thus one expects that octyl glutathione would react to a greater extent in 90 h than would butyl glutathione, and that butyl glutathione would be expected to react to a greater extent than methyl glutathione. In carbonate buffer, this was found to be the case (Table 1). NTA, which is a much smaller molecule than the glutathione tripeptides and which has three negative charges at pH 10.5, is more polar than the S-alkylglutathiones and, therefore, would be expected to be immobilized to a lesser extent in a given time period. As seen in Table 1, the percentage of NTA immobilized from solution was less than that for any of the glutathiones. The concentration of NTA in the initial solution was approximately 3-fold greater than for any of the glutathiones, yet, under these more severe conditions only 2% of the available ligand could be immobilized in 90 h. Except for methyl glutathione, the concentration of carbonate buffer had little effect on the amount of small ligand immobilized. For methyl glutathione, as the salt concentration decreased, the amount of ligand immobilized increased. It is unclear to us why methyl glutathione should act differently from the other ligands.

The effect of potassium phosphate concentration on the extent of immobilization is dramatically different from the effect of potassium carbonate concentration (Table 1). For all three of the S-alkylglutathiones, as phosphate concentration increases, the amount of ligand immobilized also increases. Increasing phosphate concentration is

known to increase hydrophobic interactions [6]. As noted previously [1,2], the increasing phosphate concentration causes a salt-induced hydrophobic association between the small affinity ligand and the stationary phase. This association then leads to an increase in reaction rate resulting in more efficient immobilization. Phosphate at 0.5 *M*, however, does not appear to be more effective than carbonate at the same concentration. For the highly polar ligand NTA, the differences in the salt type and phosphate concentration seem to have little effect. The increase in the extent of immobilization of NTA when comparing phosphate and carbonate is at best minimal with perhaps a slight advantage in using phosphate. Increasing the phosphate concentration had very little effect. Apparently for very hydrophilic ligands, such as NTA, the phosphate concentrations examined in this study are not sufficient to induce a hydrophobic interaction with the stationary phase.

At 2 *M* potassium phosphate for the three S-alkylglutathiones and at 1 *M* potassium phosphate for butyl and octyl glutathione, a maximum of 80 to 100 μmol/g of immobilized ligand is approached. The limiting factor seems not to be the total amount of epoxy groups on the HEMA since from the titration data this value was found to be 140 μmol/g. A more likely source of the limitation in the amount immobilized is due to the competing hydrolysis of the epoxy groups during the immobilization reaction. In the absence of any ligand, 38% of the epoxy groups hydrolyze leaving a total of 83 μmol/g of epoxy groups. The amount of ligand immobilized at 90 h would be a function of the rate of immobilization balanced against the rate of hydrolysis. For NTA at any phosphate concentration, the limiting factor would be the rate of immobilization and not the rate of hydrolysis. For the S-alkylglutathiones at higher phosphate concentrations, the limit of reaction is probably a function of the rate of epoxide hydrolysis. For immobilization in carbonate buffer, the extent of immobilization for any of the ligands investigated here is a function of reaction rate only.

As a general rule for the immobilization of small molecules through reaction of their amino groups with epoxy activated supports, a greater efficiency of reaction will be seen using increased concentrations of potassium phosphate. Increasing concentrations of carbonate buffer are not effective in increasing immobilization of small ligands. For very polar ligands such as NTA, increasing the concentration of neither phosphate nor carbonate is effective in increasing immobilization efficiency. For cases where the efficiency of immobilization is low, such as in the case of NTA, it may be necessary to extend the time of reaction. Extending the time of reaction does have limits, however. Although the rate of hydrolysis is slow, it can in some cases be limiting as to the final amount of ligand immobilized. Another alternative is to increase the amount of ligand in the starting solution so as to increase the rate of the immobilization reaction.

To produce a nickel affinity column of optimum coverage for the separation of a particular histidine-tagged protein, the strategy of increasing the initial concentration of NTA was adopted. The concentration of NTA in the initial solution was increased until a packing of approximately 40 μmol of NTA per gram of HEMA was obtained. The protein purified in this work was a recombinant histidine-tagged polypeptide of protein kinase C θ which had been cloned in our laboratories. Because of the potential instability of the polypeptide, we performed frequent small purifications for use in other assays. To accomplish this, we used a small HPLC column (30×2.1 mm) using the protocol listed in Fig. 3. With a column of this size, approximately 0.72 mg of the histidine-tagged polypeptide could be purified in one run. Crude extract was loaded and then washed with approximately 200 column volumes of 200 m*M* sodium phosphate buffer (pH 8.0). A gradient of 0–50 m*M* imidazole in 200 m*M* phosphate (pH 8.0) was then run over a 20-min period to elute proteins weakly complexed to the nickel ligand. A large amount of protein from the crude extract is weakly bound to the nickel chelate phase and eluted by imidazole (Fig. 3). After elution of the weakly bound proteins, the concentration of the imidazole is increased to 500 m*M*. During this rapid increase of the imidazole concentration, the polypeptide fragment is eluted in a narrow peak (peak 1, Fig. 3). This eluted material was routinely found to be greater than 90% pure by sodium dodecyl sulfate–polyacrylamide gel electrophoresis. One column was generally useful for five injections of 3 ml of crude supernatant containing the protein kinase C peptide. New columns were introduced whenever system

pressure exceeded 3000 p.s.i. Using such a small column (3 cm×2.1 mm) was useful because of the repeated number of small-scale purifications necessary for a study of this particular protein. A disadvantage of such a small column size is the limited lifetime resulting from the development of high back pressure. Column lifetime can be increased by using columns of greater diameter (4.6 mm or more) at the sacrifice of recovering more dilute purified material or having to purify larger amounts of protein for each run.

The kinetics for the desorption of the histidine-tagged polypeptide from the nickel chelate column is slow. Doubling the particle size had no practical effect on the volume of the polypeptide peak. The column described here is based on a 10-μm HEMA particle. A column based on a 20-μm particle was also prepared and gave results similar to those shown in Fig. 3. To elute the polypeptide in the smallest volume possible, a very rapid increase in imidazole concentration from 50 to 500 m*M* was required. Shallower imidazole gradients resulted in much broader peaks for the polypeptide.

The main advantages of using a nickel chelate phase based on an HPLC particle versus the same phase based on soft gels was thus not an increase in efficiency, but a decrease in the duration of the purification process and a purer product. An entire HPLC purification could be done in 50 min and, after injection, the only manual operation necessary was collection of the material. With soft gels, the purification process took nearly 2 h and required a great deal of operator attention. When isolating large amounts of material, however, the difference between HPLC and classical soft gel chromatography is not of great consequence. When making numerous small-scale purifications, the ease and convenience of HPLC methodology is invaluable. Because the pressure stability of HPLC particles is much greater than those of soft gels, higher flow-rates can be tolerated by the HPLC column which allows greater wash volumes to be used in a given time period. This leads to purer protein preparations. In this purification, for instance, 200 column volumes were used to wash the column before elution of the desired product with imidazole.

Although this work describes techniques for the efficient immobilization of small ligands, other considerations must be taken into account to produce a successful affinity phase. In particular, the density of affinity ligands on the stationary phase must be optimized. Too low a density leads to poor binding of protein with possible losses during the washing steps, while too high a density leads to poor recoveries during the elution step. Unfortunately the optimum density must be found empirically and depends upon factors such as absolute affinity, number of binding sites per protein, and physical properties of the stationary phase. Rates of reaction of small ligands will vary with the different phases. For instance the reaction of mercaptoacetic acid with the HEMA phase was much slower than with Sepharose-4B. Control of salt concentration and salt type will make this empirical process easier.

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