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Protein purification using combined streptavidin (or avidin)-Sepharose and thiopropyl-Sepharose affinity chromatography

Franck Desarnaud, Jacky Marie, Renée Larguier, Colette Lombard, Serge Jard and Jean-Claude Bonnafous

Centre CNRS–INSERM de Pharmacologie–Endocrinologie, Rue de la Cardonille, 34094 Montpellier Cedex 5 (France)

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ABSTRACT

The major problem usually encountered in the application of the (strept)avidin–biotin system to the purification of proteins (or other biological molecules) lies in the difficult reversion of the interaction between immobilized (strept)avidin and the adsorbed biotinylated protein. Among the proposed solutions is the selective biotinylation of the entity to be purified by a disulphide-containing biotinylated reagent which allows its recovery from (strept)avidin gels by dithiothreitol (DTT) treatment. As emphasized by the example of angiotensin II receptor purification, achieved using this strategy, optimum reduction of this disulphide bridge may require improvement of its accessibility using denaturating agents such as sodium dodecyl sulphate or urea. However, these agents release important amounts of (strept)avidin. Two general ways of solving this problem are proposed. One solution takes advantage of the absence of cysteine in the streptavidin sequence: the protein to be purified is selectively readsorbed to thiopropyl-Sepharose through the thiol function generated on DTT cleavage of the biotinylated reagent. The other solution is an empirical approach to make possible the use of avidin, which possesses cysteine residues: combined avidin-Sepharose and thiopropyl-Sepharose chromatography proved efficient when carried out in the presence of urea as denaturing agent.

INTRODUCTION

The so-called avidin–biotin system has been used during the last decade for the detection and purification of biomolecules. The principle consists in selective biotinylation of either the protein to be detected or purified or a ligand which interacts with this protein, followed by interaction of the biotinylated entity with derivatized or immobilized avidin or streptavidin [1–5]. Among the most striking examples are the numerous attempts to purify hormone receptors, which generally represent a small fraction of proteins and therefore require highly selective procedures for their isolation. In some instances hormone biotinylation has been used as a convenient way for its controlled immobilization on avidin or streptavidin gels, thus allowing the development of classical direct affinity chromatography [6–9].

More interestingly, hormone receptors have been covalently labelled with a biotinylated hormone before adsorption to immobilized avidin [10,11]; this type of indirect affinity chromatography is required when protein solubilization is accompanied by a loss of ligand binding. This situation includes our own work on angiotensin II (AII) receptor purification [12], which will be commented upon in this paper. In these instances, the extremely high affinity of biotin for avidin raised problems for the recovery of hormone–receptor covalent complexes. Several ways of overcoming this difficulty have been pro-

Correspondence to: Dr. J.-C. Bonnafous, Centre CNRS–INSERM de Pharmacologie–Endocrinologie, Rue de la Cardonille, 34094 Montpellier Cedex 5, France.

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posed: a first possibility is to use biotin derivatives which display decreased affinity for avidin (dethiobiotin) [10] or pH-sensitive affinity (iminobiotin) [13]. Another possibility is to use biotinylated ligands possessing a disulphide bridge, as exemplified by the work of Shimkus and co-workers [14–16] and Roseman *et al.* [17], who used Bio–S–S–dUTP (Bio = biotinyl) for nucleosome or transcriptionaly active DNA purification.

Our work on AII receptor purification was based on the photolabelling of the receptor with biotinylated azido probes and the adsorption of solubilized probe–receptor complexes on (strept)avidin gels [12]. In this instance iminobiotin-containing probes were not suitable as they displayed too high non-specific binding to starting rat liver membranes. Successful experiments were carried out using synthetic probes containing a cleavable disulphide bridge; these probes were obtained by reaction of an azido AII derivative with the commercially available NHS–SS–Bio [14–18]. Efficient receptor recovery by dithiothreitol (DTT) required the presence of sodium dodecyl sulphate (SDS), the major effect of which was to increase the accessibility of the disulphide bridge of the probe [12]. However, SDS released avidin or streptavidin from the affinity gels, so that these proteins were in large excess over the protein to be purified. We present in this paper quantitative data demonstrating the possibility of eliminating released streptavidin through thiopropyl-Sepharose chromatography. We also envisaged the possibility of using avidin gels and to reduce released avidin in the two affinity steps through the use of various denaturating agents and detergents. The potential generalization of combined streptavidin (or avidin)-Sepharose and thiopropyl-Sepharose affinity chromatography is discussed, together with the advantages of the various experimental conditions examined.

EXPERIMENTAL

Materials

The probe used for AII receptor photolabelling and purification, Bio–NH$(CH_2)_2$–S–S–$(CH_2)_2$CO–[Ala^1–Phe $(4N_3)^8$]AII [Bio–S–S–AII(N_3)], was synthesized as described previously [19]. Solutions of the azido probes were calibrated by UV spectrophotometry ($\varepsilon_{250\ nm}$ = 12 500). The radioiodinated probe and unlabelled monoiodo derivative of the probe were obtained as described previously [12,20]. Probe samples of appropriate specific radioactivities were obtained by mixing labelled and unlabelled compounds.

Biotin, avidin and streptavidin were obtained from Sigma, [^{125}I]streptavidin from Amersham, cyanogen bromide-activated Sepharose 4B and thiopropyl-Sepharose 6B from Pharmacia–LKB, YM 30 Diaflo membranes and Centricon P30 microconcentrators from Amicon, Triton X-100 (octylphenol polyethylene glycol ether) from Pierce, 3-[(3-cholamidopropyl)dimethylammonio]-1-propanesulphonate (CHAPS) from Boehringer and SDS from Bio-Rad Labs.

Electrophoresis reagents were obtained from Serva and Bio-Rad Labs. and a low-molecular-mass standard kit (14 400–97 400) from Pharmacia-LKB.

AII receptor purification

AII receptor was purified from rat liver plasma membranes using the biotinylated photoactivatable probe as described previously [12]; when applied to lower starting receptor amounts (20–100 pmol), the two affinity steps were carried out in batchwise procedures, without significant changes in their yields. Combined (strept)avidin-Sepharose and thiopropyl-Sepharose chromatography were achieved under two types of experimental conditions: apart from previously established conditions which are based on the use of DTT in the presence of SDS [12] (see details below, conditions A), some experiments involved urea (see below, conditions B) as denaturing agent in Triton X-100 or CHAPS as detergents.

Preparation of immobilized streptavidin or succinoylavidin

The preparation of avidin-Sepharose (1 mg of protein/ml of gel) and succinylation of immobilized avidin were carried out according to Finn *et al.* [6]. The succinylation step, introduced to reduce the basic character of avidin and its resulting non-specific binding to proteins [3,6], can be omitted in the preparation of streptavidin-Sepharose (1 mg/ml of gel) [3].

[^{125}I]Avidin was obtained using the chloramine T method: avidin (75 μg) in 150 μl of 50 m*M* phos-

phate buffer (pH 7.4) was iodinated with 1 mCi of $Na^{125}I$ and 40 μg of chloramine T. Radioiodinated avidin and streptavidin were diluted with unlabelled proteins so as to obtain specific radioactivities of 4.0 and 2.9 μCi/mg respectively, and immobilized as indicated above.

Behaviour of streptavidin and avidin released from affinity gels

Determinations of avidin and streptavidin contaminations achieved using immobilized [I^{125}]avidin and [I^{125}]streptavidin.

In order to mimic conditions usually applied for AII receptor purification, 30 ml of hydroxyapatite elution buffer [0.3 *M* phosphate (pH 6.0), 0.5% Triton X-100, 1% SDS, 5 m*M* EDTA, 1 m*M* phenylmethylsulphonyl fluoride (PMSF) and 0.5 m*M* N-ethylmaleimide (NEM)] were applied to a 3-ml column of radioactive gel initially equilibrated in the same buffer without SDS. After an initial wash in the presence of 1% SDS, the column was thoroughly rinsed with several buffers containing 0.5% Triton X-100, as described previously [12]. At this stage several conditions were checked for elution from these gels and for the thiopropyl-Sepharose chromatography of the (strep)avidin eluates:

Conditions A. Elution was achieved by gentle agitation (15 min at room temperature) with 15 ml of 10 m*M* phosphate buffer (pH 8.0)–50 m*M* DTT, 1% SDS. The eluates were freed from most their DTT content by three successive ultrafiltration steps through YM 30 Diaflo membranes, separated by fivefold dilution with 10 m*M* phosphate buffer (pH 6.0) and 0.1% Triton X-100.

The samples were adsorbed, with gentle agitation for 2 h, on 250 μl of thiopropyl-Sepharose 6B (the pH of the sample was adjusted to 7.5–7.8). The gels were then washed with 10 m*M* phosphate buffer (pH 8.0)–0.1% SDS; elution was achieved with the same buffer supplemented with 50 m*M* DTT. The thiopropyl-Sepharose eluates were submitted to ultrafiltration in Amicon Centricon P30 microconcentrators [three cycles separated by fivefold dilution with 10 m*M* phosphate buffer (pH 8.0)–0.1% SDS].

Conditions B. In experiments involving immobilized avidin the gel was presaturated with 6 μM biotin before elution with 10 m*M* phosphate buffer (pH 8.0)–50 m*M* DTT–8 *M* urea in 0.1% Triton X-100. DTT and urea removal by ultrafiltration and sample adsorption to thiopropyl-Sepharose were carried out as for conditions A. The thiopropyl-Sepharose gel was then washed with 10 m*M* phosphate buffer (pH 8.0) containing either 0.1% SDS or 8 *M* urea in 0.1% Triton X-100 or 1% CHAPS; elutions were achieved with the same buffers supplemented with 50 m*M* DTT. The eluates were ultrafiltered as for conditions A, except that SDS was replaced with Triton X-100 or CHAPS.

SDS–polyacrylamide gel electrophoresis (PAGE)

Purified AII receptor, avidin and streptavidin were analyzed under reducing conditions by SDS-PAGE according to the method of Laemmli [21]. Samples were treated for 1 h at 30°C in a medium containing 80 m*M* Tris–HCl (pH 6.8), 2% (w/v) SDS, 10% (w/v) glycerol, 0.1 *M* DTT and bromophenol blue. A 12.5% acrylamide running gel overlaid by a 5% acrylamide stacking gel was used (electrophoresis conditions: 14–16 h, 50 V). Gels were stained using silver or Coomassie Brilliant Blue. Dried gels were autoradiographed at −80°C with Kodak XAR-5 films and intensifying screens.

RESULTS

Principle of protein purification through combined (strept)avidin-Sepharose and thiopropyl-Sepharose chromatography: the example of the angiotensin II receptor

We recently published a detailed protocol for AII receptor purification, based on covalent labelling of the membrane receptor with a biotinylated photoactivatable hormone derivative, followed by solubilization and selective adsorption of the solubilized probe–receptor complexes on immobilized streptavidin [12]; we adopted this strategy because it appeared impossible to bind AII to the solubilized receptor so that purification through classical affinity chromatography had to be ruled out; the difficulty in reversing the streptavidin–biotin interaction led us to synthesize a probe containing a disulphide bridge between biotin and angiotensin II: Bio–NH$(CH_2)_2$–S–S–$(CH_2)_2$CO–[Ala^1–$Phe(4N_3)^8$]AII.
The synthesis of this probe [19] was facilitated by the commercial availability (Pierce) of the disulphide containing biotin derivative Bio–NH$(CH_2)_2$–S–S–$(CH_2)_2$COOR (R = succinimidyl) (NHS–S–

TABLE I

BEHAVIOUR OF BIOTINYLATED AII RECEPTOR COVALENT COMPLEXES THROUGHOUT COMBINED AVIDIN OR STREPTAVIDIN-SEPHAROSE AND THIOPROPYL-SEPHAROSE CHROMATOGRAPHY

AII receptor was purified from rat liver plasma membranes using [^{125}I]Bio–S–S–AII(N_3) (initial concentration, 8 nM; specific radioactivity, 180 Ci/mmol as mentioned under Experimental. Hydroxyapatite eluates containing 0.5% Triton X-100 were adsorbed on avidin or streptavidin gels in the presence or absence of 1% SDS. Receptor adsorption to thiopropyl-Sepharose and its elution from streptavidin or avidin gels and thiopropyl-Sepharose were carried out under various conditions as described under Experimental. The values given in this table were obtained with streptavidin-Sepharose gels. Similar values were obtained with avidin-Sepharose gels.

Step	Receptor adsorption yield (%)			Receptor elution yield (%) (DTT)		
	SDS	Triton X-100	CHAPS	SDS	Urea	
					Triton X-100[a]	CHAPS
Streptavidin-Sepharose	83	71	N.D.[b]	82	81	N.D.[b]
Thiopropyl-Sepharose	66	84	91	87	75	90

[a] The elution yield was 97% in the absence of urea.
[b] N.D. = Not determined.

S–Bio), a starting compound previously used for the preparation of biotinylated nucleotides [14].

Covalent complexes specifically obtained by AII receptor labelling with the radioiodinated probe were easily adsorbed on immobilized avidin or streptavidin [12]; however, receptor recovery through DTT treatment was not completely satisfactory when carried out in Triton X-100 as detergent [12]. A first possibility of solving this problem would have been to synthesize other probes displaying increased accessibility of their disulphide bridges with respect to receptor or streptavidin steric requirements. Alternatively, we choose to try to improve accessibility of this S–S bond through the use of denaturating agents. The first experiments carried out in the presence of SDS were satisfactory in terms of receptor recovery (82% yield, Table I). However, SDS treatment released avidin or streptavidin in amounts that were unsuitable for subsequent accurate electrophoretic analysis and separation. Analysis of the streptavidin peptide sequence [22] surprisingly revealed that this protein does not contain any cysteine residue. We took advantage of this to eliminate contaminating streptavidin by selectively adsorbing the receptor to thiopropyl-Sepharose through the SH function which results from DTT cleavage of the spacer arm of the probe (see Fig. 2, Scheme 1 for illustration). The possible receptor readsorption through the thiol functions resulting from DTT reduction of its intramolecular disulphide bridges cannot be excluded; however, the thiol function of the cleaved probe, which occupies an "antennary" position, is probably the most accessible to the activated thiol functions of thiopropyl-Sepharose.

The 6000-fold purification allowed by this protocol was established by measurement of radioactive receptor and total protein content of the samples [12]. As a consequence of the initial covalent labelling of the binding site, it was not possible to characterize pharmacologically the purified receptor; the identity of the purified glycoprotein to the AII receptor was assessed by the following criteria: the specificity of labelling in purified samples has been demonstrated [12]; the purified receptor displayed an electrophoretic pattern [average molecular mass (M_r) 65 000] similar to that of starting solubilized receptor; deglycosylation of non-purified and purified receptor led to the same M_r 40 000 entity [12]. Obviously these patterns cannot result from an artifact involving contaminating streptavidin (streptavidin is not glycosylated; see also the next paragraph).

The purpose of this work was to validate this

strategy of purification (see Fig. 2 for a detailed general scheme of the procedure); pilot experiments described in the next section confirmed the prediction that thiopropyl-Sepharose chromatography is a convenient way of eliminating released steptavidin. Moreover, taking into account that streptavidin is much more expensive than avidin, an important constraint for large-scale experiments, we tried to establish conditions that would allow protein purification through combined avidin-Sepharose and thiopropyl-Sepharose chromatography with acceptable final avidin contaminations. The rational solution would have consisted in reducing avidin disulphide bridges and alkylating the resulting thiol functions, in order to suppress their ability subsequently to react with thiopropyl-Sepharose; however, it appeared impossible, consistent with the work of Green [23], who found that disulphide reduction only occurred on denaturated avidin, and that alkylation prevents renaturation. In this respect, one must mention that adsorption of biotin-tagged AII receptor to monomeric avidin gels [24] was not successful. As a consequence, we used an empirical approach which consisted in limiting the release of avidin at the first affinity step and limiting its adsorption and release at the thiopropyl-Sepharose step. Based on the fact that tetrameric avidin is fairly stable in urea [25,26] or on biotin binding [27], we checked the possibility of saturating the avidin gel with biotin prior to its DTT treatment, and used urea instead of SDS as denaturating agent to improve accessibility of the disulphide bridge of the biotinylated reagent to be cleaved.

Behaviour of streptavidin and avidin during (strept) avidin-Sepharose and thiopropyl-Sepharose chromatography

Pilot experiments consisted in the use of immobilized radioiodinated avidin and streptavidin, which allowed the easy determination of the amounts of these proteins initially released from affinity gels by denaturing agents, then subsequently adsorbed to and released from the thiopropyl-Sepharose matrix.

Streptavidin-Sepharose. In a first series of experiments we evaluated the release of avidin and streptavidin and demonstrated subsequent elimination of streptavidin, under conditions strictly identical with those established for AII receptor purification; the results, expressed in μg of protein/ml of starting gel, are given in Table II. Treatment of avidin or streptavidin gels with DTT in 1% SDS released less streptavidin (35 μg) than avidin (83 μg). To underline the importance of this contamination, it must be noted that under similar conditions, using the minimum amount of gel required for optimum receptor adsorption, the amount of eluted AII receptor was about 300 ng (a minor fraction of immobilized (strept)avidin is involved in receptor binding; for details see the next section); the intrinsic amount of released avidin or streptavidin precludes accurate electrophoretic elimination in large-scale preparations.

DTT removal by ultrafiltration through YM 30 Diaflo membranes was carried out in Triton X-100-containing buffers because this detergent unexpectedly allowed more efficient AII receptor readsorption to thiopropyl-Sepharose (84%) compared with SDS (66%) (Table I); this finding should not necessarily be extended to other proteins to be purified. Some streptavidin or avidin was eliminated in the first ultrafiltration cycles, probably by dialysis of monomers (which might cease when the Triton/SDS ratio reached values allowing subunit reassociation).

As expected, streptavidin was poorly and non-specifically adsorbed to thiopropyl-Sepharose (10%), while the bulk of avidin was specifically adsorbed (82%) and DTT eluted (93%) (Table II); the amount of recovered streptavidin (1.4 μg) can be considered as acceptable inasmuch it is exclusively found in monomeric form when treated with SDS and analysed by SDS-PAGE; Fig. 1A illustrates the absence of residual tetrameric radioiodinated streptavidin; the protein to be purified can be freed from residual contamination by electroelution, provided its molecular mass is in the suitable range (65 000 for AII receptor, see Fig. 1A).

Avidin-Sepharose. Biotin presaturation of the avidin-Sepharose gel and the use of urea instead of SDS as denaturing agent in the elution medium proved equally efficient in reducing the amount of released avidin (not shown), their combination being slightly more advantageous (Table II). Although most of the avidin contained in the ultrafiltered sample was adsorbed to thiopropyl-Sepharose, only 30% was eluted with DTT in the presence of SDS, and less than 10% with DTT in the presence of urea in Triton X-100; the interpretation is

TABLE II

BEHAVIOUR OF AVIDIN OF STREPTAVIDIN THROUGHOUT COMBINED AVIDIN OR STREPTAVIDIN-SEPHAROSE AND THIOPROPYL-SEPHAROSE CHROMATOGRAPHY

3 ml of [^{125}I]avidin (4.0 μCi/mg of protin) and [^{125}I]streptavidin (2.9 μCi/mg of protein) gels were treated under various conditions designed for AII receptor purification as described in under Experimental. The amounts of recovered avidin or streptavidin were measured in the various steps involved in the purification procedure.

Step	Streptavidin (μg/ml of starting streptavidin-Sepharose gel)	Avidin (μg/ml of starting avidin-Sepharose gel)	
	Conditions for elution from streptavidin gel	Conditions for elution from avidin gel	
	DTT–SDS	DTT–SDS	Biotin presaturation + DTT–urea–Triton X-100
Elution from avidin or streptavidin gel	35	83	13.5
YM 30 ultrafiltration	17.5	72	5.9
Thiopropyl-Sepharose adsorption	1.8	59	5.3
Thiopropyl-Sepharose elution			
DTT–SDS	1.4	55	1.6
DTT–urea–Triton X-100	N.D.[a]	N.D.[a]	0.5

[a] N.D. = Not determined.

that the disulphide bridge joining thiopropyl-Sepharose to avidin displays different DTT accessibilities under the two conditions, and that this accessibility is systematically decreased when SDS has been omitted in the preceding step. As a final result, the residual avidin contamination was lowered to a value close to, or even lower (0.5 μg/ml of starting gel, *i.e.*, less than 1/1000 of immobilized avidin) than that previously obtained for streptavidin (1.4 μg/ml of starting gel). Remaining avidin was exclusively found as its monomeric form after SDS denaturation and SDS-PAGE (Fig. 1B).

General schemes for protein purification

That conclusions drawn from the above-described control experiments should be valid for protein purification assays can be inferred from the following evidence: only 1/1000 of immobilized avidin or streptavidin is involved in the adsorption of biotinylated AII receptor complexes, as previously established in gel saturation experiments [12]; avidin or streptavidin is stabilized upon biotin binding [27]; it was confirmed, in AII receptor purification experiments, by the lack of receptor elution during SDS washing of the gels: not more than 1–1.5% (five experiments) of the adsorbed probe–receptor complexes were eluted on washing with four column volumes of buffer containing 1% SDS.

We have verified that initial replacement of SDS with urea in Triton X-100 did not have a significant effect either on AII receptor elution from avidin or streptavidin gels or on its readsorption or re-elution in the thiopropyl-Sepharose step, which emphasizes the validity of this new set of experimental conditions (various yields are given in Table I and electrophoretic patterns of the purified receptor are shown in Fig. 1C).

As a consequence, we can propose two general schemes (Fig. 2) for the purification of a protein which has been selectively derivatized with NHS–S–S–Bio or which has reacted (covalently or not covalently) with the appropriate biotinylated ligand.

Although the initial covalent labelling of the protein with the biotinylated reagent and the use of denaturing agents might not allow purified protein displaying all its native properties to be obtained,

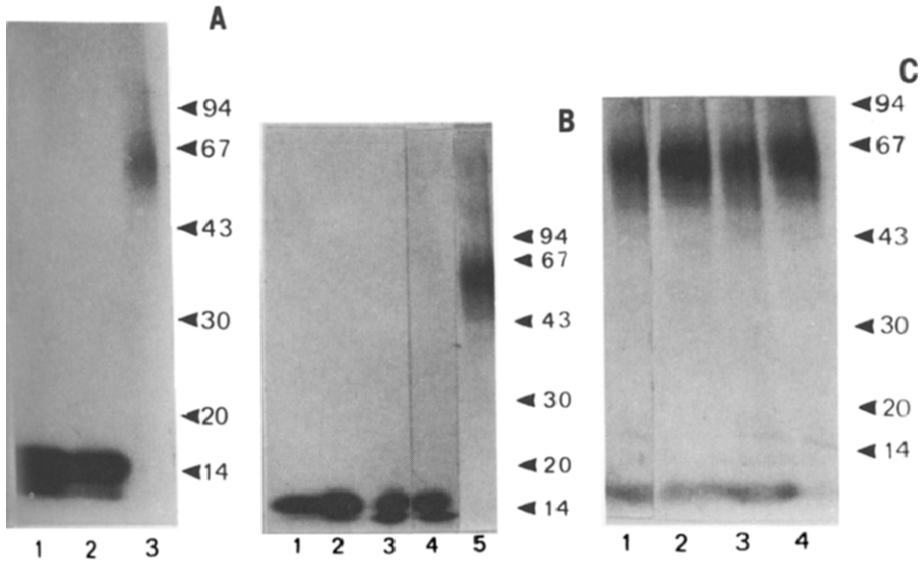

Fig. 1. Electrophoretic mobilities of purified AII receptor and streptavidin or avidin released in control experiments. A 3-ml volume of immobilized [^{125}I]streptavidin-Sepharose (2.9 μCi/mg protein) or [^{125}I]avidin (4.0 μCi/mg) was treated under conditions usually applied in AII receptor purification as described under Experimental. AII receptor was purified from rat liver plasma membranes using [^{125}I]Bio–S–S–AII(N_3) (initial concentration, 6–8 n*M*; specific radioactivity, 180–1800 Ci/mmol) as mentioned under Experimental. AII receptor, released [^{125}I]streptavidin and [^{125}I]avidin were analysed by SDS-PAGE (12.5% acrylamide gel) and autoradiography. (A) Released [^{125}I]streptavidin (2.8 μg); lane 1 = DTT elution from [^{125}I]streptavidin-Sepharose in the presence of SDS; lane 2 = DTT elution from [^{125}I]streptavidin-Sepharose and thiopropyl-Sepharose in the presence of SDS (see Experimental, conditions A); lane 3 = purified AII receptor (51 fmol). (B) Released [^{125}I]avidin (1.2 μg): lane 1 = DTT elution from [^{125}I]avidin-Sepharose in the presence of SDS; lane 2 = DTT elution from [^{125}I]avidin-Sepharose and thiopropyl-Sepharose in the presence of SDS (see Experimental, conditions A); lane 3 = DTT elution from biotin-presaturated [^{125}I]avidin-Sepharose in the presence of urea in Triton X-100; lane 4 = DTT elution from biotin-presaturated [^{125}I]avidin-Sepharose and thiopropyl-Sepharose in the presence of urea in Triton X-100 (see Experimental, conditions B); lane 5 = purified AII receptor (38 fmol). (C) Electrophoretic pattern of AII receptor purified under various elution conditions from streptavidin-Sepharose and thiopropyl-Sepharose. Purified AII receptor (1–2 fmol, 1800 Ci/mmol) was analysed by SDS-PAGE (12.5% acrylamide gel) and by autoradiography. Lane 1 = DTT elution from streptavidin-Sepharose and thiopropyl-Sepharose in the presence of SDS (see Experimental, conditions A); lanes 2–4 = DTT elution from biotin-presaturated streptavidin-Sepharose in the presence of urea, followed by DTT elution from thiopropyl-Sepharose in the presence of either SDS (lane 2), or urea in Triton X-100 (lane 3) or CHAPS (lane 4) (see Experimental, conditions B). Numbers on the right are molecular masses of protein standards ($\times 10^{-3}$).

many structural applications are consistent with these constraints (antibody production, obtaining partial peptide sequences from purified intact protein or fragments for cloning or mapping studies, recognition of the protein or fragments by specific antibodies, etc).

Combinations between the two purification schemes can easily be imagined; for instance, although these modifications have not been checked for streptavidin gels, one can postulate that biotin saturation followed by the use of urea might further improve the elimination of contaminating streptavidin.

Efficient AII receptor elution from thiopropyl-Sepharose could be achieved in Triton X-100-containing buffers in the absence of urea (Table I). Interestingly, AII receptor could be efficiently adsorbed to thiopropyl-Sepharose in CHAPS as detergent and DTT eluted in presence of urea in CHAPS (Table I). This emphasizes the various possible conditions for obtaining purified proteins essentially freed from contaminating avidin or streptavidin, and the various media in which purified samples can be finally handled.

DISCUSSION

Among the numerous applications of the avidin–

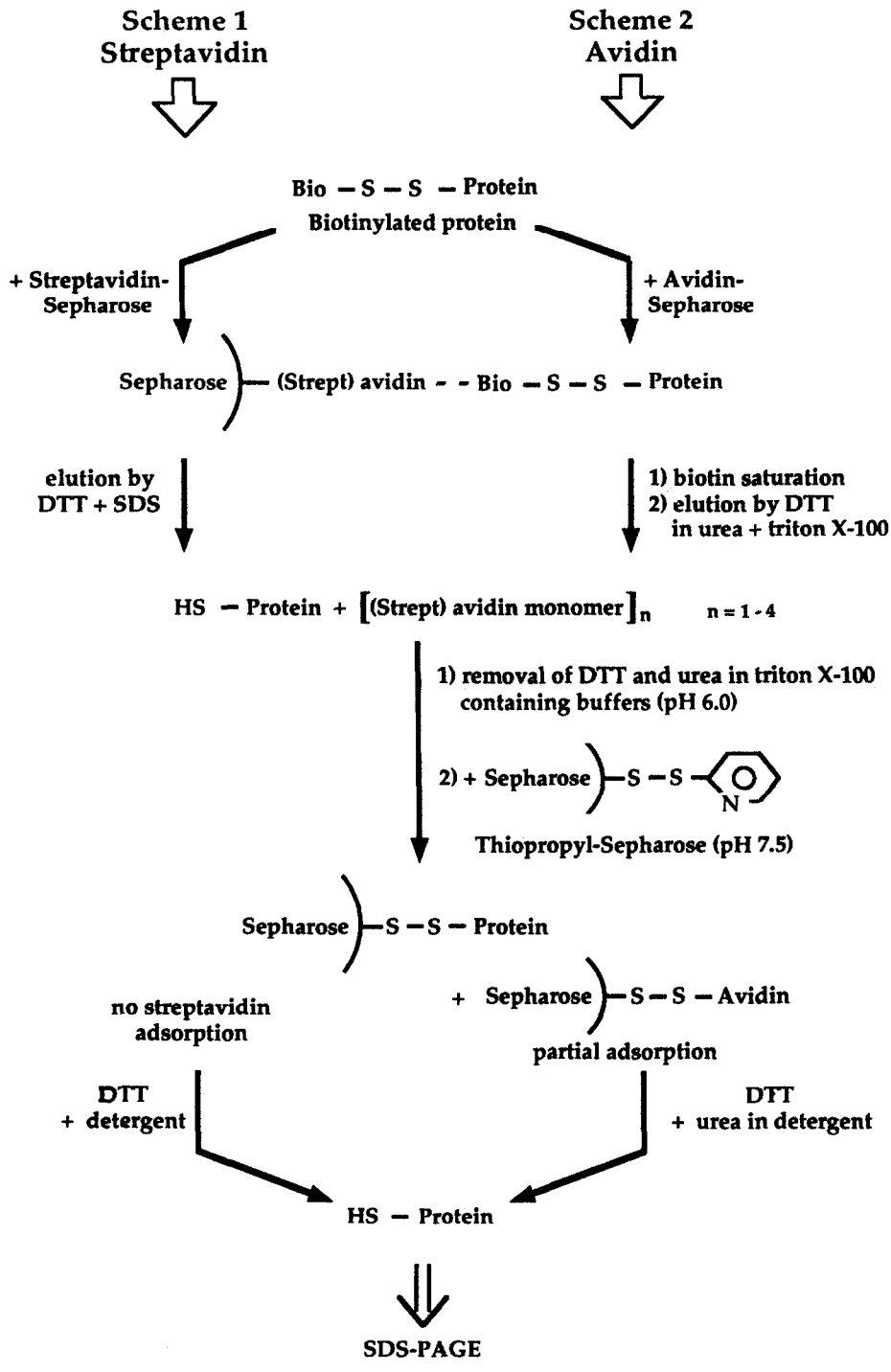
Scheme 1
Streptavidin
Scheme 2
Avidin
Bio — S — S — Protein
Biotinylated protein
+ Streptavidin-Sepharose
+ Avidin-Sepharose
Sepharose — (Strept) avidin - - Bio — S — S — Protein
elution by DTT + SDS
1) biotin saturation
2) elution by DTT in urea + triton X-100
HS — Protein + [(Strept) avidin monomer]n n = 1 - 4
1) removal of DTT and urea in triton X-100 containing buffers (pH 6.0)
2) + Sepharose — S — S
N
Thiopropyl-Sepharose (pH 7.5)
Sepharose — S — S — Protein
+ Sepharose — S — S — Avidin
no streptavidin adsorption
partial adsorption
DTT + detergent
DTT + urea in detergent
HS — Protein
SDS-PAGE

Fig. 2.

biotin system, those involving detection and quantitative evaluations of biotinylated entities using appropriate avidin derivatives have been the most straightforward [1–5]; many examples refer to the study of interactions between biomolecules. The wide use of this system essentially results from the extremely high affinity of avidin for biotin, which generally allows high residual affinity when biotin has been linked to one of the interacting partners. Conversely, purification purposes, which involve reversal of the biotin–avidin interaction, have been achieved with difficulty. Solutions to these problems based on the use of chemically modified biotin (iminobiotin [13], dethiobiotin [10]) cannot be generalized without raising new problems; for instance, our strateygy for AII receptor purification [12] could not be developed with iminobiotin hormone derivatives which display prohibitive non-specific binding to membranes; the use of a dethiobiotin–ACTH derivative for receptor purification [10] did not eliminate the need for denaturing treatment in the recovery step from affinity gels, which induced release of important amounts of avidin. Another possibility is to use biotin derivatives containing a disulphide function which can be reduced under mild conditions [14–18]. The data obtained with several Bio–S–S–dUTP derivatives emphasize that optimization of the length of the linkers joining biotin to dUTP was required to favour accessibility of the S–S bond to DTT [16].

The biotinylated photoactivatable probe that we have designed for AII receptor purification [12] was obtained by reaction of azido AII derivatives with the commercially available NHS–S–S–Bio [19]: covalent complexes between this probe and rat liver receptor could be efficiently adsorbed to (strept)avidin gels, under conditions where premature cleavage of the probe was suppressed by NEM alkylation of the thiol functions of the membrane preparations [12]; however, high recoveries of these complexes by DTT treatment required the accessibility of the probe S–S bond to SDS to be improved; this latter induced the release of unacceptable amounts of avidin or streptavidin. Instead of searching for an ideal synthetic probe possessing an accessible S–S bond, we tried to find convenient ways for avidin or streptavidin elimination. We could propose two kinds of solutions. The most rational one takes advantage of the fortuitous absence of cysteine in the streptavidin protein sequence [22]; this made possible selective readsorption of the protein to be purified through the thiol function generated on DTT cleavage of the spacer arm (additional readsorption through the other thiol functions of the protein, if any, constitutes a favourable factor); as predicted, residual streptavidin was lowered to acceptable values. The second solution is an empirical approach to make possible the use of avidin, which possesses cysteine residues [28] but is much less expensive than streptavidin; the proposed procedure involves limitation of initial avidin release by biotin saturation of avidin-Sepharose before DTT elution and the use of urea (in Triton X-100) instead of SDS to favour this elution. These initial elution conditions greatly influence the behaviour of avidin in the recovery step from thiopropyl-Sepharose gel; the use of DTT in the presence of urea leaves the bulk of adsorbed avidin unreleased, as a probable result of poor accessibility of the disulphide bridge involved, under these less denaturating conditions.

Fig. 2. Combined (strept)avidin-Sepharose and thiopropyl-Sepharose affinity chromatography applied to protein purification. The proposed purification schemes are applied to a protein which has previously been selectively biotinylated using the commercially available reagent NHS–S–S–Bio (or another synthetic reagent possessing a disulphide bridge). The biotinylated protein is adsorbed to streptavidin-Sepharose or avidin-Sepharose, then recovered by DTT treatment in the presence of either SDS (Scheme 1) or urea in Triton X-100 (Scheme 2); these denaturing agents favour the accessibility of the disulphide bridge of the biotinylated reagent, but at the same time release non-negligible amounts of streptavidin or avidin subunits (evaluation of the degree of subunit reassociation is beyond the scope of the paper); biotin presaturation of the gel and the use of urea greatly reduce the amount of released avidin (see text). Most of the DTT and urea are eliminated by ultrafiltration–dilution cycles carried out at pH 6.0 to avoid reoxidation of the thiol function generated on the spacer arm of the biotinylated reagent; this thiol function allows readsorption of the protein to an activated thiol-matrix: thiopropyl-Sepharose (after pH readjustment to 7.5). Released streptavidin, devoid of any cysteine residue, is not adsorbed (Scheme 1); although significantly adsorbed to thiopropyl-Sepharose through its thiol functions, avidin (possibly reassociated into subunits) is poorly eluted by DTT + urea, probably because of reduced accessibility of the disulphide bridge which joins it to thiopropyl-Sepharose (Scheme 2). The protein is once again efficiently recovered by DTT treatment (see the example of AII receptor commented upon in Table I), before possible analysis and further purification by SDS-PAGE. Although not having been systematically checked, combinations between these two schemes can be imagined.

The small amounts of (strept)avidin remaining after application of one of the proposed solutions can be further eliminated, if required, by SDS-PAGE.

As urea exerts little denaturing effect on avidin and streptavidin [25,26], its effect on AII receptor recovery from affinity gels results from an increase in the accessibility of the probe S–S bond through receptor denaturation; this emphasizes that avoiding denaturing agents would imply the chemical design of biotin derivatives possessing an S–S bond in the right position with respect to their receptor anchorage sites; as a consequence, generalization of the use of a given biotinylated reagent would not necessarily be possible as the steric requirements would obviously depend on the protein to be purified.

As a result of our investigations, initiated by difficulties encountered for AII receptor purification, we can propose the possible extension of the following purification scheme: selective biotinylation of the entity to be purified by a classical disulphide bridge-containing reagent, and development of combined streptavidin (or avidin)-Sepharose and thiopropyl-Sepharose chromatography. The latter step, introduced to eliminate (strept)avidin, may provide by itself additional purification; moreover, it offers the possibility of finally handling the purified entity in appropriate detergents, when required.

The reported experiments refer to conditions adapted to membrane protein purification; there is little doubt that the proposed ways of eliminating (strept)avidin might be applied to soluble proteins even when DTT recovery of the entity to be purified would not require the use of denaturating agents. In addition, leakage of ligands immobilized to cyanogen bromide-activated agarose cannot always be ignored; thiopropyl-Sepharose might constitute a convenient tool for eliminating released multimeric or monomeric streptavidin.

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