POSSIBLE PITFALLS IN THE IDENTIFICATION OF GLYCOPHORIN-BINDING PROTEINS OF PLASMODIUM FALCIPARUM

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During the asexual blood stage of malarial infection, the parasite invades red blood cells, matures to the schizont form, and ruptures to release numerous merozoites, which can invade further uninfected red cells. During invasion of human red cells by merozoites of Plasmodium falciparum, glycophorin is thought to act as a recognition and attachment site for the merozoite (1, 2). Several investigators have therefore attempted to identify malarial glycophorin-binding proteins using glycophorin coupled to a solid support (3–6).

Glycophorin-coupled CNBr-Sepharose was found to bind 140,000 and 35,000 M_r [$^{35}$S]methionine-labeled schizont proteins of a Ugandan Palo Alto isolate. These proteins were reported to elute with N-acetylglucosamine, suggesting a lectin-like interaction (3). By contrast, glycophorin-coupled aminoethyl-BioGel (AE-BioGel) bound heat-stable parasite proteins of 130,000 and 155,000 M_r, which were present in schizonts, merozoites, and culture supernatants of an FCR-3 isolate and which were selectively labeled by [$^3$H]proline (4). By Western blotting with a human monoclonal antibody, other investigators have detected 155,000, 135,000, 120,000, and 65,000 M_r polypeptides in culture supernatants which bind to glycophorin-coupled AE-BioGel (5). These four glycophorin-binding proteins carried antigenic structures related to the tandemly repeated portion of the amino acid sequence of the 155,000 M_r ring-infected erythrocyte surface antigen (RESA) (5).

Here we report a major problem encountered when using AE-BioGel as a support matrix for glycophorin affinity chromatography, which was not taken into account in previous studies. Under some conditions, large amounts of [$^3$H]-proline-labeled parasite proteins bind to AE-BioGel in the absence of coupled glycophorin. With the FCR-3 isolate, we found that binding was highly selective for proteins of 130,000 and 155,000 M_r. Binding of these proteins is apparently related to the highly positively charged nature of AE-BioGel, and is very sensitive

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to ionic conditions and to the degree of equilibration of the column with buffer. While these data do not exclude the possibility that some of the parasite antigens identified might also bind specifically to glycophorin, they suggest that information obtained using glycophorin-coupled AE-BioGel must be interpreted with caution.

Materials and Methods

Collection of Biosynthetically Labeled Culture Supernatants. *P. falciparum* (FCR-3) was cultured in vitro using standard techniques (7). Synchronous cultures at 10–20% parasitemia, which had been trypsinized at ring stage to remove most glycophorin (2), were grown to mid-schizont stage, washed with proline and glutamine-free medium and biosynthetically labeled for 2.5 h at 37°C and 10% hematocrit in proline and glutamine-free medium supplemented with 0.1 mCi/ml L-2,3-[3H]proline (30–60 Ci/mmol; Amersham International, Amersham, United Kingdom). After labeling, the parasites, usually containing ~20% of the added radioactivity, were washed with serum-free medium and used to prepare schizont lysate or culture supernatant.

Culture supernatant was prepared by culturing the labeled parasites at 10% hematocrit in medium containing only 2% (vol/vol) human serum, for 4–8 h until ~75% of the schizonts had ruptured. The final culture was centrifuged at 1,000 g for 5 min, and protease inhibitors [aprotinin, antipain, chymostatin, and leupeptin (all at 0.01 mg/ml); N-tosyl-L-phenylalanine chloromethylketone (0.1 mM), N-acetyl-L-lysine chloromethylketone (0.2 mM), phenylmethylsulfonylfluoride (1 mM), 2,4,6-phenanthroline (1 mM), p-hydroxymercuribenzoate (1 mM), and iodoacetamide (5 mM); Sigma Chemical Co., Poole, United Kingdom] were added to the supernatant. The supernatant was then centrifuged again at 1,000 g for 5 min, passed through a BioDyne A nylon membrane (pore size 1.2 μm; Gallenkamp, London, United Kingdom) (8), centrifuged at 11,000 g for 5 min, and finally centrifuged at 20,000 g for 1 h. To prepare heated culture supernatant, supernatant from the 11,000 g spin was heated at 100°C for 10 min, cooled on ice, centrifuged at 3,000 g for 10 min, supplemented with a further 2% (vol/vol) serum, heated at 100°C for 10 min, cooled on ice, supplemented with 0.01 mg/ml aprotinin, and centrifuged at 11,000 g for 5 min, and at 20,000 g for 1 h.

Schizont lysate was prepared by resuspending the washed, labeled schizont culture at 10% hematocrit in PBS, pH 8.0, containing 1% (wt/vol) NP-40 (BDH Ltd., Poole, United Kingdom), 0.1% (wt/vol) sodium deoxycholate (Sigma Chemical Co.), 1 mM ZnCl₂, 0.02 mg/ml DNase I (Boehringer Corp. Ltd., Lewes, United Kingdom), and protease inhibitors as for culture supernatants, but excluding phenylmethylsulfonyl fluoride, iodoacetamide, and p-hydroxymercuribenzoate (4, 9, 10). After 1 min, phenylmethylsulfonyl fluoride, iodoacetamide and p-hydroxymercuribenzoate were also added and the lysate was centrifuged at 11,000 g for 5 min and at 20,000 g for 1 h to remove insoluble material.

Preparation of Glycophorin-coupled AE-BioGel. Glycophorin was prepared from Dodge ghosts of outdated normal human red cells as described (11), except that, before gel filtration, 0.1% (wt/vol) Ammonyx-LO (Millmaster-Onxy Ltd., Oldham, United Kingdom) was added to the sample and that a Sephacryl S400 (Pharmacia Fine Chemicals) column was used with a 5 mM NaH₂PO₄ buffer, pH 8.0, containing 25 mM NaCl, 1 mM EDTA, and 0.1% (wt/vol) Ammonyx-LO. Fractions containing protein detectable at 290 nm were analyzed by SDS-PAGE and periodate-Schiff staining (12). Fractions containing predominantly glycophorin A were pooled and further characterized by amino acid analysis (Table I). Concentrations of glycophorin solutions were determined by amino acid analysis, allowing for the carbohydrate content, and/or by absorbance at 280 nm.

For coupling, 0.4 g AE-BioGel was swollen for 2 h at room temperature with 50 ml H₂O, adjusted to pH 6 with HCl, washed twice with 50 ml H₂O, and adjusted to pH 5. 15 mg 1-ethyl-3-(3-dimethylaminopropyl)carbodiimide was added to either 3 ml H₂O or 3 ml glycophorin (0.8 mg/ml) in H₂O at pH 5. After 5 min, the solutions were added to separate 0.2-g aliquots of swollen AE-BioGel and rotated for 16 h at room temperature with adjustment of pH to 5 for the first 2 h using 0.1 M HCl. After coupling, the columns
were washed alternately with 0.1 M sodium phosphate buffer, pH 8, or sodium acetate buffer, pH 4, both containing 1 M NaCl, and finally with 15 washes of 50 ml PBS. Coupling was verified by monitoring the A290 of coupling supernatants and high-salt washes (80% coupling), and qualitatively by immunofluorescence. For the latter assay, blank and glycophorin-coupled beads were incubated and washed three times with 15 volumes of FCS and analyzed under the fluorescence microscope after treatment with R10 antigycohpiorin mAb (kindly donated by Drs. D. J. Anstee and M. J. A. Tanner, Bristol, United Kingdom) and fluorescein-labeled goat anti-mouse immunoglobulin (Sigma Chemical Co.).

Binding to and Elution from AE-BioGel. AE-BioGel (BioRad Laboratories, Watford, United Kingdom) was either swollen in two volumes PBS according to the manufacturer’s instructions (unequilibrated AE-BioGel), or equilibrated by washing 15 times with 10 volumes PBS, allowing at least 20 min per wash (equilibrated AE-BioGel). All buffers used for elution contained protease inhibitors as listed above. 0.6 ml packed acrylamide beads were rocked with 1 ml schizont lysate or 2 ml culture supernatant for 1 h at room temperature, washed four times with 10 ml PBS, and eluted sequentially as described (4), with 0.3 ml 2 × PBS, 10 ml PBS, 0.5 ml 0.5 M NaCl in PBS, 10 ml PBS, 0.3 ml 2% SDS in PBS, 10 ml PBS, and 0.3 ml 4% SDS in PBS at 100°C for 10 min. Radioactivity in samples of beads and eluted fractions was counted after bleaching for 16 h with 0.1 ml 1 M NaOH and 0.3 ml 0.5% H2O2 (13), and then adding 10 ml Liquiscint (National Diagnostics, Somerville, NJ) and 0.025 ml glacial acetic acid.

SDS-PAGE. SDS-PAGE on 7.5% gels was carried out according to Laemmli (14), except that the Tris HCl concentration in the separating gel was 0.75 M; in the stacking gel, 0.063 M. 10% gels were run similarly except that the acrylamide/bisacrylamide ratio was 30:0.4 instead of 30:0.8. All samples were run under reducing conditions. Gels were stained and processed for fluorography (10). M, were determined relative to [14C]-labeled

| Amino acid | Residues per 131 residues |
|------------|---------------------------|
|            | Found*                    | Expected† |
| Asx        | 10.0 ± 0.6                | 8         |
| Thr        | 13.7 ± 1.8                | 15        |
| Ser        | 18.1 ± 0.5                | 18.5      |
| Glx        | 17.2 ± 1.1                | 14.5      |
| Pro        | 10.0 ± 1.4                | 10        |
| Gly        | 5.9 ± 0.1                 | 5.5       |
| Ala        | 7.4 ± 0.8                 | 6         |
| Val        | 8.2 ± 2.6                 | 11        |
| Met        | 1.9 ± 0.1                 | 2         |
| Ile        | 9.8 ± 1.3                 | 11        |
| Leu        | 7.7 ± 0.8                 | 7.5       |
| Tyr        | 3.6 ± 2.1                 | 4         |
| Phe        | 1.6 ± 0.2                 | 2         |
| His        | 5.1 ± 0.4                 | 5         |
| Lys        | 5.5 ± 0.2                 | 5         |
| Arg        | 5.9 ± 0.0                 | 6         |

* Samples of glycophorin prepared as described in Materials and Methods were hydrolyzed under vacuum with 6 M HCl for 16 h. Hydrolysates were analyzed on a Beckman I19CL analyzer. Data shown are means ± SD of two preparations. No attempt has been made to correct for degradation of labile amino acids or for the presence of amino sugars.

† Expected values for MN glycophorin A, deduced from the sequence of the 131-residue polypeptide chain (25).
protein standards (Amersham Corp.): Myosin (200 kD), phosphorylase b (92.5 kD), BSA (69 kD), ovalbumin (46 kD), and carbonic anhydrase (30 kD).

**Western Blotting.** Proteins from SDS-polyacrylamide gels were electrophoretically transferred onto nitrocellulose as described (15) at 100 mA constant current for 16 h. Protein binding sites on the nitrocellulose were then blocked by incubation for 1 h in 50 mM Tris HCl, 150 mM NaCl, 0.02% NaN₃, pH 7.5, containing 20% (vol/vol) FCS. After blocking, the nitrocellulose filters were incubated for 90 min at 37°C with appropriate dilutions of antibody preparations in blocking buffer containing 0.1% (wt/vol) Tween-20 (Sigma Chemical Co.). They were then washed 10 times with 50 mM Tris Cl, 150 mM NaCl, 0.1% (wt/vol) Tween-20, pH 7.5. Staphylococcal protein A (Pharmacia Ltd., Milton Keynes, United Kingdom) was iodinated using Iodogen (Pierce Ltd., Cambridge, United Kingdom) as described (14), to a specific activity of 20 mCi/mg, and was incubated with the washed filters for 1 h at 37°C at a dilution of 25 ng/ml in blocking buffer containing 0.1% (wt/vol) Tween-20. The filters were then washed 10 times with washing buffer and mounted on DuPont Lightning Plus intensifying screens for autoradiography with pre-flashed Fuji x-ray film at −70°C.

Human affinity-purified antibodies against *P. falciparum* Ag 23 (GBP130) (17, 18) (used at 1:50 dilution) were kindly donated by Dr. Robin Anders, Walter and Eliza Hall Institute of Medical Research, Melbourne, Australia. Pooled Gambian human immune serum was kindly donated by Dr. Kevin Marsh, Nuffield Dept. of Clinical Medicine. Prestained proteins (Bethesda Research Labs Ltd., Cambridge, United Kingdom) were used as molecular mass standards (200, 92.5, 68, 43, and 25.7 kD).

**Results**

**Binding Characteristics of AE-BioGel and BioGel P30.** The [³H]proline-labeled FCR-3 culture supernatants we obtained contained ~30% TCA-precipitable label. ~40% of the TCA-precipitable radioactivity was heat-stable. Incubation of culture supernatants with equilibrated AE-BioGel followed by extensive washes with PBS resulted in binding of ~20% of the total radioactivity applied. By contrast, the use of uncharged materials such as BioGel P30, Sephadex G75, or Sephacryl S300 resulted in binding of <1% of radioactivity applied. Similar results were obtained with schizont lysates. Of the radioactivity that could be eluted from AE-BioGel, the majority eluted with 0.5 M NaCl in PBS, although a small proportion was recovered in the subsequent 4% SDS fraction. SDS treatment caused the beads to collapse and aggregate, so that it was difficult to determine the amount of radioactivity remaining on the beads after boiling with SDS. However, it appeared that a significant fraction of the radioactivity remained bound to AE-BioGel even upon boiling with SDS. Of the small amount of radioactivity bound to uncharged BioGel P30, a greater proportion eluted with SDS than with NaCl.

**Heat-stable [³H]Proline-labeled FCR-3 Proteins.** SDS-PAGE analysis of [³H]-proline-labeled whole culture supernatants revealed two prominent heat-stable (HS) bands in the 120,000–160,000 Mₗ region, denoted HS130 and HS155 (Fig. 1B, lane 2). These two bands were characterized by a diffuse outline and varied in relative proportion in different preparations as noted previously (4). Two bands, HL150 and HL160, running close to and on either side of HS155 on 7.5% gels were not heat-stable (compare Fig. 1A, lane 1 with lane 2, and Fig. 1B, lane 1 with lane 2).

**[³H]Proline-labeled FCR-3 Proteins Binding to AE-BioGel and Glycophorin-coupled AE-BioGel.** 0.5 M NaCl eluates from AE-BioGel were analyzed by SDS-PAGE on 7.5% gels. When either culture supernatants or schizont lysates had been
applied, bands of 130,000 and 155,000 $M_r$, were the major species detected (Fig. 1B, lane 3, and Fig. 1A, lane 5). The bands from heated culture supernatants or schizont lysates had the characteristic diffuse outline of the heat-stable 130,000 and 155,000 $M_r$ bands seen in whole culture supernatants.

Binding and elution of the 130,000 and 155,000 $M_r$ components was highly selective (Fig. 1A, lane 5), although other proteins binding to AE-BioGel could be detected by overexposure of the fluorograph of the gel of the NaCl eluates (Fig. 1B, lane 3) or by boiling the final AE-BioGel beads with SDS-PAGE sample buffer rather than 4% SDS. Several proteins, including those of 130,000 and 155,000 $M_r$, were almost quantitatively removed from culture supernatants applied to AE-BioGel, whereas some other bands did not bind (Fig. 2, lanes 1–4). The extent of removal was more difficult to assess with schizont lysates because of the multiplicity of bands (Fig. 2, lanes 5–7).

The $[^3]$H proline-labeled eluates from AE-BioGel looked the same whether they eluted from blank or glycophorin-coupled AE-BioGel columns (Fig. 1B,
FIGURE 2. Absorption of \(^{3}H\)proline-labeled proteins by AE-BioGel. 7.5% acrylamide gel of \(^{3}H\)proline-labeled FCR-3 samples. Lane 1, culture supernatant; 2, supernatant absorbed with uncharged BioGel P30; 3, supernatant absorbed with AE-BioGel; 4, supernatant supplemented with 20% serum and then absorbed with AE-BioGel; 5, schizont lysate; 6, schizont lysate absorbed with BioGel P30; 7, schizont lysate supplemented with 20% serum and then absorbed with AE-BioGel.

lanes 3 and 4). \(^{3}H\)proline-labeled HS130 and HS155 also eluted from columns to which heated culture supernatant had been applied (Fig. 1B, lanes 5 and 6).

Conditions Governing Binding to AE-BioGel. In the experiments described above, the AE-BioGel was washed 15 times with 10 volumes of PBS after swelling. However, the extent of washing of the beads and the nature of the sample dramatically affected the amount of parasite proteins bound and eluted. In one series of experiments, AE-BioGel was swollen with two volumes PBS according to the manufacturer's instructions, but not washed, and was compared with equilibrated AE-BioGel. When schizont lysates were applied to the unequilibrated column, the NaCl eluates contained <10% of the radioactivity of those from an equilibrated AE-BioGel column (Fig. 3, compare lanes 3 and 5). Using culture supernatants, with one batch of unequilibrated AE-BioGel (obtained from RioRad Laboratories, Watford, United Kingdom), 0.5 M NaCl still eluted 130,000 and 155,000 M, proteins, but with another batch (obtained from BioRad Laboratories, Richmond, CA) the eluates contained <50% of the radioactivity eluted from equilibrated AE-BioGel. Supernatant collected from the beads after swelling was able to inhibit binding of proteins to equilibrated AE-BioGel (data not shown).

Adding samples of different pH to either equilibrated or unequilibrated columns also affected the type and amount of parasite proteins in the NaCl eluates. For instance, with AE-BioGel from BioRad-Watford and schizont lysate, adjustment of the sample pH to 5.0 before application to the equilibrated column
The effect of column equilibration and pH on AE-BioGel binding. 10% acrylamide gel of [3H]proline-labeled FCR-3 samples. Lane 1, [3H]proline-labeled schizont lysate; 2, NaCl eluate from equilibrated AE-BioGel to which schizont lysate adjusted to pH 5 had been applied; 3, NaCl eluate from equilibrated AE-BioGel to which schizont lysate at pH 8 had been applied; 4, NaCl eluate from unequilibrated AE-BioGel to which schizont lysate at pH 5 had been applied; 5, NaCl eluate from unequilibrated AE-BioGel to which schizont lysate at pH 8 had been applied.

resulted in a 40% reduction in the radioactivity in the NaCl eluate and loss of the 130 kD band (Fig. 3, compare lanes 2 and 3) compared to the pH 8.0 sample. With the unequilibrated column, the pH 5.0 sample similarly showed the 155,000 M<sub>r</sub> but not the 130,000 M<sub>r</sub> band in the NaCl eluate (Fig. 3, lane 4), but the pH 8.0 sample did not give any bands in the NaCl eluate. This NaCl eluate contained <10% of the radioactivity of the equivalent sample from the equilibrated column (Fig. 3, compare lanes 3 and 5).

Supplementing the culture supernatant or schizont lysate with 20% vol/vol human serum had no detectable effect on the amount or type of [3H]proline-labeled parasite proteins bound to the equilibrated column (data not shown).

Equilibrated DEAE-Sephadex bound only about twofold as much radioactivity as the same volume of equilibrated AE-BioGel. The NaCl eluate from DEAE-Sephadex appeared similar to that from AE-BioGel in being enriched in the 130,000 and 155,000 M<sub>r</sub> proteins of FCR-3, although other proteins were also present (Fig. 4).

Antigenic Analysis of FCR-3 Proteins Binding to AE-BioGel. Western blots of culture supernatants with human affinity-purified antibodies to Ag 23, kindly provided by Dr. Robin Anders, showed a series of bands, with the two major ones in the 90,000–110,000 M<sub>r</sub> region. This material was no longer detected in culture supernatants after application to equilibrated AE-BioGel (Fig. 5, lane 5). Several bands, including the two major bands, appeared in the NaCl eluate in
FIGURE 4. Comparison of AE-BioGel and DEAE-Sephadex binding proteins. [$^3$H]proline-labeled FCR-3 schizont lysate was applied to either equilibrated AE-BioGel or DEAE-Sephadex, and the NaCl eluates were analyzed on a 10% acrylamide gel. Lane 1, NaCl eluate from AE-BioGel; 2, NaCl eluate from DEAE-Sephadex.

the case of equilibrated AE-BioGel (Fig. 5, lane 3), but little if any of this material could be detected in the NaCl eluate from BioGel P30 (Fig. 5, lane 4).

Pooled Gambian immune human serum, kindly provided by Dr. Kevin Marsh, reacted with a number of bands in FCR-3 culture supernatants by western blot. Equilibrated AE-BioGel selectively removed specific bands in the 100,000–200,000 $M_r$ range from culture supernatants (Fig. 6, lane 5). Several bands in this range were detected in the NaCl eluate from AE-BioGel (Fig. 6, lane 3). With BioGel P30, no bands were removed from culture supernatants or detected in the NaCl eluate (Fig. 6, lanes 6 and 4).

Serological analysis with selected human antisera from Gambia showed that the S antigens of seven isolates tested all bound to AE-BioGel in the absence of coupled glycophorin. Elution with 0.5 M NaCl gave bands with the molecular masses and serological identities that divided them into three groups: (a) FCR-3, 155,000 $M_r$; FVO, 155,000 $M_r$; D4, 155,000 $M_r$; (b) Lf13, 180,000 $M_r$; LFi, 200,000 $M_r$; and (c) T-26, 220,000 $M_r$; and CDC-1, 220,000 $M_r$ (data not shown).
Discussion

The above results reveal a potential pitfall in the use of AE-BioGel as a support matrix for glycophorin affinity chromatography. Equilibrated AE-BioGel selectively and quantitatively binds a number of proteins from culture supernatants and schizont lysates of the FCR-3 isolate of *P. falciparum*, including proteins of 130,000 and 155,000 M_. This binding is likely to be related to the high charge density of AE-BioGel, which compares well with conventional anion exchangers (Table II). This hypothesis would explain why 20% of applied radioactivity bound to AE-BioGel, as compared with <1% for uncharged materials. It is also consistent with the sensitivity of AE-BioGel binding to ionic conditions, such as varying methods for washing the beads, differences in pH, and the presence of 0.5 M NaCl, and with the binding of the 130,000 and 155,000 M_ proteins to DEAE-Sephadex.

Upon coupling proteins to AE-BioGel using 1-ethyl-3(3-dimethylaminopropyl)carbodiimide, the amino groups to which proteins are coupled are chemically blocked and therefore would be expected no longer to participate in anion exchange, while the remaining amino groups remain chemically unchanged (Fig. 7). In principle, therefore, coupling of proteins could reduce the ion-exchange capacity of AE-BioGel. However, in practice, as the amino group concentration
FIGURE 6. Immunoblot with Gambian human immune serum. 7.5% acrylamide gel of FCR-3 samples blotted onto nitrocellulose and probed with Gambian human immune serum and 125I-protein A. Lane 1, FCR-3 culture supernatant; 2, heated culture supernatant; 3, NaCl eluate from AE-BioGel to which culture supernatant had been applied; 4, NaCl eluate from BioGel P30 to which culture supernatant had been applied; 5, culture supernatant absorbed with AE-BioGel; 6, culture supernatant absorbed with BioGel P30.

TABLE II

| Material             | Exchange capacity (dry gel) | Settled volume | Exchange capacity (wet gel) | Hemoglobin capacity |
|----------------------|----------------------------|----------------|-----------------------------|---------------------|
|                      | mEq/g  | m/l/g | mEq/ml | mg/ml |
| DEAE Bio-Gel A       | 0.020  |       | 0.020  | 45*   |
| Cellex D (standard capacity) | 0.7    | 8    | 0.088  |        |
| AE-BioGel            | 1.5    | 14   | 0.107  |        |
| DEAE-Sephacel        | 1.4    |      | 0.095-0.135 |        |
| DEAE-Sephadex        | 3.5    | 18‡  | 0.194  |        |
|                      | 36§    |      | 0.097  | 139    |

Figures in the table are quoted or calculated from information in manufacturers' catalogues or product labels. Exchange capacity is the amount of charged and potentially charged groups in a specified amount of ion exchanger. Hemoglobin capacity is the amount of hemoglobin (M, 69,000) reversibly bound by ion exchanger. DEAE Bio-Gel A, Cellex D, and AE-Bio-Gel P150 are proprietary products of BioRad Laboratories Inc., Richmond, CA. DEAE-Sephacel and DEAE-Sephadex are proprietary products of Pharmacia Fine Chemicals AB, Uppsala, Sweden.

* Hemoglobin capacity is quoted for 5 mM Tris Cl buffer, pH 8.6, and the volume of DEAE Bio-Gel A is known to be constant over a wide range of pH and ionic strength.

‡ Wet exchange capacity of DEAE-Sephadex A50 was calculated on the basis of pH 7.6 Tris Cl buffer containing 150 mM NaCl.

§ Figure quoted is for 10 mM Tris Cl buffer, pH 8.0, the buffer in which hemoglobin capacity was measured.
in swollen AE-BioGel is 107 mM according to the manufacturer's specification, it would be difficult to have any significant effect on the ion-exchange capacity of AE-BioGel simply by coupling a protein ligand. For instance, to couple 1 mg/ml glycophorin through one carboxyl group per molecule would block chemically only 0.03 mM amino groups, and even if the glycophorin molecules could be unfolded and coupled through every carboxyl group on the molecule this would only block 1.5 mM amino groups. We cannot estimate the steric blocking of amino groups that might be achieved by coupling proteins at 1 mg/ml concentrations. However, it may be noted that ion exchangers with similar capacities to AE-BioGel are able to bind reversibly two orders of magnitude higher concentrations of proteins than this (e.g., see hemoglobin capacities in Table II). These considerations suggest that highly charged matrices such as AE-BioGel are more suitable for coupling small molecules, where high coupling ratios can be achieved. When proteins at 1 mg/ml concentrations have been coupled to AE-BioGel, it seems likely that ion-exchange interactions with the support would occur in addition to affinity interactions with the coupled protein. Our results on binding of [3H]proline-labeled parasite proteins to blank and glycophorin-coupled columns are consistent with this view.

It is well known that ion exchange can give variable results unless the column is fully equilibrated with buffer and the sample is applied in the same buffer. This rigorous procedure is not always necessary for affinity chromatography. Our results highlight that AE-BioGel pretreated in different ways has very different binding properties. Briefly, while several factors, such as pH, could affect binding, our most striking finding was that inadequate ionic equilibration of AE-BioGel can cause a dramatic decrease in binding of parasite proteins, presumably because of the presence of particular counter ions on the dry resin as supplied. Extensive washing with PBS is necessary for equilibration. It was also noteworthy that different proteins showed different sensitivity to pH and equilibration in their binding to AE-BioGel (Fig. 3). It is a corollary of these observations that it is hard to compare results obtained with blank and glyco-
Acidic Sequences in Selected Antigens of P. falciparum

| Antigen                  | pI  | Main repeat          | Net charge per repeat |
|--------------------------|-----|----------------------|-----------------------|
| S-Antigen, FC27          | 4.2 | PAKASQGGLED          | -1                    |
| RESA, FC27, and NF7      |     | EENV (EHDA)          | -2                    |
| NF7                      |     | DDEHVEEP'TVA         | -4                    |
| Ag 23/GBP 130            | 5.0 | 50 amino acids       | -4 to -7              |

Net charge was calculated from published sequences assuming D = -1; E = -1; K = +1; R = +1. Also, for the purpose of this table, H was taken to carry a charge of +1 so as to allow for the maximum possible neutralization of negative charges. Data are obtained from previous studies (17–19, 22, 23).

Glycophorin-coupled AE-BioGel columns unless ion-exchange conditions are rigorously controlled.

Glycophorin-coupled AE-BioGel has been reported to bind several proteins in the 120,000–160,000 M₉ range. One protein is the major heat-stable protein labeled by [³H]proline or [³H]glycine in culture supernatants. In FCR-3 this protein is 155,000 M₉ (4). Another protein that has been identified as binding to glycophorin-coupled AE-BioGel is GBP130 or Ag 23, which is a heat-stable antigen present in culture supernatants but not in merozoites, variously described as 110,000 or 130,000 M₉, and conserved in all parasite isolates tested (17–20). The third protein identified is RESA or Pf155, which is 155,000 M₉, present in culture supernatants, conserved between isolates, and usually heat-stable (5, 18, 21). All three proteins are acidic or contain acidic repeat sequences (Table III), and so the possibility of their binding by anion exchange rather than or in addition to glycophorin binding must be considered.

The major [³H]proline-labeled heat stable bands in FCR-3 culture supernatants were HS155 and HS130. These properties indicate that these proteins are S-antigens of FCR-3. The 155,000 M₉ protein is known to be an FCR-3 S-antigen (R. J. M. Wilson, unpublished observations), and the smaller band is most likely to be derived from the larger by proteolysis. This feature of doublet bands of varying relative intensity in different preparations has been commonly observed for S-antigens (22–24). Furthermore, we found that S-antigens of each of a panel of seven isolates of P. falciparum bind to AE-BioGel. The removal of the putative S-antigen of FCR-3 by AE-BioGel is almost quantitative under the conditions used (Fig. 2), and binding was quite robust to changes in conditions (Fig. 3). Because the protein is already bound quantitatively by blank AE-BioGel, the identical behavior of glycophorin-coupled AE-BioGel (Fig. 1B) is to be expected and neither indicates nor disproves any glycophorin binding activity.

Our immunoblots for Ag 23 in culture supernatants are consistent with those described previously (20) in showing two major bands in the 90,000–110,000 M₉ range. These bands bound to AE-BioGel and were present in the NaCl eluate. We detected no Ag 23 antigenic activity remaining in the culture supernatant after absorption with AE-BioGel.

AE-BioGel has been used as a solid support for glycophorin affinity chromatography in an attempt to avoid two potential problems of using CNBr-Sepharose. First, parasite glycophorin-binding proteins may require glycophorin to be cou-
Plasmodium falciparum glycophorin-binding proteins

pled in a particular orientation for the appropriate binding site to be accessible. Second, because a lectin-like interaction has been postulated for the invasion process \((2, 3)\), an acrylamide- rather than a carbohydrate-based support would certainly be preferable. However, our results demonstrate that the use of the acrylamide-based coupling matrix AE-BioGel in its turn introduces a major problem associated with the anion-exchange properties of the support. All three proteins hitherto identified as binding to glycophorin-AE-BioGel are acidic and therefore must be considered as having the potential to bind to blank AE-BioGel under appropriate conditions, and in the case of S-antigen and bands with Ag 23 antigenic activity, we were able to demonstrate binding. The selective binding of these 130,000 and 155,000 \(M_r\) proteins to AE-BioGel does not exclude the possibility that these or other proteins may also bind specifically to glycophorin. However, it would be hard to study specific binding to coupled glycophorin when the support matrix makes such a large contribution to the total binding to the column, and when binding to the support matrix is so sensitive to ionic conditions. Therefore, while it is still possible that these or other proteins of similar molecular mass bind to glycophorin, our results urge the need for further studies to examine the specificity of glycophorin binding using a variety of different support matrices to allow for the problems inherent in each.

Summary

Plasmodium falciparum proteins that bind to the putative erythrocyte receptor (glycophorin) have been identified in several laboratories by their ability to bind to glycophorin immobilized on aminoethyl-BioGel (AE-BioGel). We here report that several parasite proteins bind to AE-BioGel in the absence of coupled glycophorin. Binding is apparently due to the strong ion-exchange properties of the matrix, and is sensitive to ionic conditions such as the degree of equilibration of the matrix and the pH. The parasite proteins that bind to the blank column under appropriate conditions include proteins with the serological activities of S-antigen and Ag 23, which also bind to glycophorin-coupled AE-BioGel. In the light of these results, the glycophorin-binding specificity of these and other proteins reported to bind to glycophorin-coupled AE-BioGel will have to be reevaluated, preferably using a different support matrix.

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