Disulfide Bond Involvement in the Maintenance of the Cryptic Nature of the Cross-Reacting Determinant of Metacyclic Forms of *Trypanosoma congolense*†

Wallace R. Fish,* Cecilia W. Muriuki, Anthony M. Muthiani, Dennis J. Grab, and John D. Lonsdale-Eccles
International Laboratory for Research on Animal Diseases, P.O. Box 30709, Nairobi, Kenya
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ABSTRACT: The variable surface glycoprotein (VSG) of African trypanosomes possesses a 1,2-dimyristoylglycosylphosphatidylinositol at the carboxy terminus. Cleavage of the 1,2-dimyristoylglycerol (1,2-DMG) moiety from the VSG reportedly results in a higher apparent molecular mass and an increased binding of antibodies against the "cross-reacting determinant" (CRD), a cryptic epitope present on most VSGs. Using metacyclic forms of *Trypanosoma congolense*, we show that the processes involved are more complex than heretofore presumed and that the removal of the 1,2-DMG moiety may not be necessary for binding of anti-CRD antibodies (RxCRD). Among other findings, we observe the following: (1) in sonicated samples of trypanosomes metabolically labeled with [^{3}H]myristate, the binding of RxCRD on Western blots is coincident with bands containing labeled (membrane form) VSGs; (2) disulfide reduction of trypanosome sonicates suffices to promote RxCRD binding in the presence or absence of inhibitors of a glycosylphosphatidylinositol-specific phospholipase C; (3) trypanosomes directly solubilized in detergents show quantitative and qualitative differences in RxCRD binding which depend upon the detergent used and the order of addition of disulfide reducing agents. We conclude that the binding of RxCRD to *T. congolense* metacyclic VSGs depends upon the degree of unfolding of the molecule and is clearly a complex, multistep process in which structural changes and disulfide reduction play pivotal roles.

Bloodstream forms of African trypanosomes possess a cell-surface coat composed of a single, variable glycoprotein called VSG (the variable surface glycoprotein).[1] This VSG, like several other proteins bearing covalent lipid constituents that have recently come under close scrutiny, is anchored to the cell membrane via a glycosylphosphatidylinositol (GPI) moiety found at the carboxy terminus (C-terminus). The phospholipid portion of the anchor contains 1,2-dimyristoylglycerol [1,2-DMG; see Ferguson and Williams (1988) for review and complete references]. Also located in the C-terminus is a complex moiety, responsible for some immunological cross-reactivity among VSGs of the same and different species of trypanosomes. This is called the cross-reacting determinant (CRD; Barbet & McGuire, 1978). Groups or domains implicated in affecting the binding of anti-CRD antibodies (RxCRD) include galactose, glucosamine, inositol cyclic 1,2-phosphate (Zamze et al., 1988), and 1,2-DMG. Proteins from many lower and higher eukaryotes also contain a carboxy-terminal GPI group, and some of these molecules have been shown to react with RxCRD [see Ferguson and Williams, (1988)].

The CRD has been reported to be unavailable for RxCRD binding unless the 1,2-DMG moiety has been removed (Turner, 1985), and increased binding of RxCRD to VSG has been correlated with the action of a GPI-specific phospholipase C (GPI-PLC) on the C-terminus of "membrane form" VSG (mfVSG; Cardoso de Almeida & Turner, 1983). The release of 1,2-DMG from mfVSG also yields a "soluble form" VSG (sVSG) with a slightly increased apparent molecular mass (M_r). The trypanosome GPI-PLC that catalyzes this reaction is apparently influenced by metal ions, detergents, and thiol reactive reagents (Cardoso de Almeida et al., 1984; Bulow & Overath, 1985; Gurnett et al., 1986; Hereld et al., 1986).

Metacyclic forms (the infective form transmitted by the tsetse fly; Ross et al., 1987) of *Trypanosoma congolense*, as well as bloodstream forms, possess a GPI anchor and an enzyme capable of cleaving 1,2-DMG. However, during our studies on the VSGs of these parasites, we noticed several differences between our results and those of others. We therefore investigated these differences further and found that, under certain conditions, increased RxCRD binding can occur simply by the addition of reducing agents and need not involve the removal of 1,2-DMG.

Materials and Methods

Materials. Biotinylated secondary antibodies, streptavidin-biotinylated horseradish peroxidase complex (SBHRP), nitrocellulose sheets (HyBond C), all radioactive materials, and X-ray film were from Amersham (U.K.). Urea was ultrapure grade from Schwarz/Mann. All SDS–PAGE components were from Pierce, Bio-Rad, or Fluka (Switzerland). Protease inhibitors were purchased from Cambridge Research Biochemicals (U.K.), Percoll was from Pharmacia (Sweden), and DEAE-cellulose (DE-53) was from Whatman (U.K.). Tween 20 was Surfact Amp grade (Pierce), and $CoCl_2 \cdot 6H_2O$ was Puratronic Grade (Alfa). All other chemicals were of analytical reagent grade or better.

Preparation of Trypanosomes. Metacyclic forms of *Trypanosoma* (*Nannomonas*) *congolense*, clone IL 3000, were propagated and isolated by slight modifications of methods

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[1] Abbreviations: BSA, bovine serum albumin; C-terminus, carboxy terminus; CHAPS, 3-[(3-cholamidopropyl)dimethylammonio]-1-propanesulfonate; CHAPSO, 3-[(3-cholamidopropyl)dimethylammonio]-2-hydroxy-1-propanesulfonate; CRD, cross-reacting determinant; 1,2-DMG, 1,2-dimyristoylglycerol; DTT, dithiothreitol; GPI, glycosylphosphatidylinositol; GPI-PLC, glycosylphosphatidylinositol-specific phospholipase C; kDa, kilodaltons; LSC, liquid scintillation counting; VSG, variable surface glycoprotein; mfVSG, "membrane form" VSG; sVSG, "soluble form" VSG; PBSGH, phosphate-buffered saline containing glucose and hypoxanthine; pCMBS, *p*-(chloromercuri)-benzenesulfonate; RxCRD, anti-CRD antibody(ies); SDS–PAGE, sodium dodecyl sulfate–polyacrylamide gel electrophoresis; SBHRP, streptavidin–biotinylated horseradish peroxidase complex; Zn^{2+}, $ZnCl_2$; ZW-314, Zwittergent 3-14; M_r, relative molecular mass.

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described in Gray et al. (1987). Trypanosomes were concentrated and washed by centrifugation (1000*g*, 10 min, no brake, 4 °C) in phosphate-buffered saline (43.5 mM sodium phosphate, pH 7.4, and 29 mM NaCl) containing 1% (w/v) glucose and 100 μM hypoxanthine (PBSGH; Lonsdale-Eccles & Grab, 1987). Procyclic forms were grown in medium similar to that used in the propagation of metacyclic cultures but contained only 10% (v/v) fetal bovine serum and 10 μg of hemin/mL. *T. congolense* IL 3000 bloodstream form trypanosomes were isolated from the blood of infected Sprague-Dawley rats on Percoll gradients (Grab & Bwayo, 1983) containing 100 μM hypoxanthine. These trypanosomes were further purified on DEAE-cellulose (Lanham & Godfrey, 1970) using buffers containing 100 μM hypoxanthine, and washed with PBSGH.

Labeling of Trypanosomes with [^{3}H]Myristic Acid. [^{3}H]Myristate/bovine serum albumin (BSA) was prepared according to Voorheis (1980) and was dissolved in sufficient medium (as above except with 2%, v/v, fetal bovine serum) to give 100 μCi of [^{3}H]myristate/mL. Metacyclic trypanosomes were suspended in this solution at 2×10^7 organisms/mL. After 3 h at 27 °C, the labeled trypanosomes were collected by centrifugation and washed 3 times with PBSGH.

Production of RxCRD IgG and Assay for GPI-PLC. Rabbit immunoglobulin G specific for the CRD was obtained as described by Grab et al. (1984). Briefly, the IgG fraction of antiserum raised against purified *T. brucei brucei* ILTat 1.1 sVSG was isolated on protein A–Sepharose. Specific RxCRD was isolated from this IgG fraction by affinity chromatography on a purified *T. b. brucei* MITat 1.6 sVSG Sepharose column. All experiments shown in this paper were done with this batch of affinity-purified IgG.

Sonicates of *T. congolense* metacyclic forms were analyzed for the presence of GPI-PLC by measuring the release of 1,2-[^{3}H]DMG from [^{3}H]myristate-labeled *T. b. brucei* VSG, as described by Grab et al. (1987). Sonicates were prepared either in the presence of the chosen inhibitors or the inhibitors were added after sonication. When present, dithiothreitol (DTT) was added to 100 mM.

Preparation of Trypanosome Lysates, Polyacrylamide Gel Electrophoresis, and Western Blotting. Trypanosomes were suspended in PBSGH containing the protease inhibitors antipain, chymostatin, leupeptin, and *trans*-epoxysuccinyl-L-leucylamido-4-guanidinobutane (Lonsdale-Eccles & Mpimbaza, 1986) at 40 μg/mL each inhibitor. The preparations were then sonicated (continuous cooling on ice, 50 W, 5 s, three bursts separated by 1 min; Branson Model 2000 with microprobe, FRG), or lysed by detergent, or lysed by a combination of hypotonic conditions and freeze–thawing (Ross et al., 1987). Membrane and supernatant fractions were prepared by centrifugation in a Beckman Airfuge using a fixed-angle rotor (100000*g*, 1 h, 4 °C). Samples were solubilized for sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS–PAGE) by heating (10 min, 100 °C) immediately after the addition of boiling sample buffer to a final concentration of 62 mM Tris-HCl (pH 6.8), 2% (w/v) SDS, 7.5% (v/v) glycerol, and 0.02% (w/v) bromphenol blue. When present, a reducing agent, usually DTT, was added to 100 mM. Some whole metacyclic sonicates were reduced and cysteine residues alkylated with iodoacetamide. The method of Waxdal et al. (1968), as described in Fontana and Gross (1986), was followed except the sonicate had SDS added to 2% (w/v) and then heated (100 °C, 10 min) prior to dialysis into alkylation buffer in which urea (8 M) was substituted for guanidine hydrochloride. 2×10^7 or 5×10^6 trypanosomes were loaded for Coomassie Brilliant Blue staining or Western blotting, respectively. Samples were separated on 7.5–17.5% gradient acrylamide slab (1 mm thick) gels containing 0.1% (w/v) SDS (Maizel, 1971) with a 3% acrylamide stacking gel. After electrophoresis, proteins either were stained with Coomassie Brilliant Blue or were transferred to nitrocellulose sheets.

Western blotting was performed at 100 V (3 h, 4 °C) as described by Burnette (1981) except the transfer buffer contained only 0.01% (w/v) SDS (Nielsen et al., 1982). After transfer, the nitrocellulose was blocked overnight (4 °C) in 20 mM sodium phosphate (pH 7.5), 0.25 M NaCl, and 0.02% (w/v) sodium azide containing 3% (w/v) BSA. Buffers containing antibodies, probes, or substrates also contained 1% (w/v) BSA. All BSA-containing buffers were filtered (0.20-μm pore size). Application of probes (1 h) and washes (10 min) were under constant agitation. After blocking, blots were exposed to 8 M urea (Erickson et al., 1982) containing 2% (w/v) BSA (60 °C, 1 h). Between urea treatment and probing with RxCRD, blots were washed 3 times with 20 mM Tris-HCl (pH 8.1), 0.25 M NaCl, and 0.05% (v/v) Tween 20 (rinse buffer 1), then once with 0.3% (v/v) Tween 20 (Batteiger et al., 1982) in the same buffer, and, finally, 3 times in rinse buffer 1, prior to application of the primary antibody.

After further washing with rinse buffer 1, blots were probed with biotinylated secondary antibodies in 20 mM sodium phosphate (pH 7.5), 0.25 M NaCl, and 0.05% (v/v) Tween 20 (rinse buffer 2). After six washes in rinse buffer 2, the blot was probed (30 min) with SBHRP followed by three washes of rinse buffer 2. The blot was then incubated with 25 mg of diaminobenzidine/mL, 15 mg $CoCl_2 \cdot 6H_2O$/mL, and 0.1% (w/v) H_2O_2 (as per Amersham, U.K., handbook) in the same buffer containing 1% (w/v) BSA. Development proceeded rapidly (within 1–2 min for all blots) and was terminated by three rapid changes of buffer containing 0.1% (w/v) sodium azide.

Biotinylated molecular mass standards were prepared (Della-Penna et al., 1986) from a Bio-Rad product and were calibrated against ^{14}C-radiolabeled standards. All molecular masses shown in figures are given in kilodaltons (kDa).

Liquid Scintillation Counting of Isotope-Containing Blots. Sonicates of [^{3}H]myristate-labeled metacyclic forms were blotted and probed with RxCRD. Some blots were probed with SBHRP. Others were probed with ^{35}S-labeled streptavidin (0.1 μCi of streptavidin/cm^2 of nitrocellulose). The washed and dried blots were autoradiographed to locate the labeled bands of VSG. Wide cellophane tape was then applied to these nitrocellulose sheets to provide a solid support. Horizontal slices were cut, dissolved in 1 mL of acetone, and counted for ^{3}H and ^{35}S in 20 mL of Aquasol 2. Samples were corrected for differential quench, channel spillover, and the background on adjacent pieces of the blot.

Results

Figure 1 shows a Coomassie Blue stained gel of the proteins of *T. congolense* IL 3000 bloodstream, metacyclic and procyclic forms. All samples showed multiple bands in the region of VSG (~49–57 kDa; see below), thus precluding a simple assignment of any of these protein bands as VSG. *T. congolense* metacyclics showed no obvious differences in band mobility, regardless of the method of preparation, although nonreduced samples did show a slight reduction in the M_r of bands in the region of VSGs. These changes were difficult to assign to any particular band. This, and the natural heterogeneity of metacyclic VSGs [see also Luckins et al. (1986)], necessitated an alternative method of VSG identification.

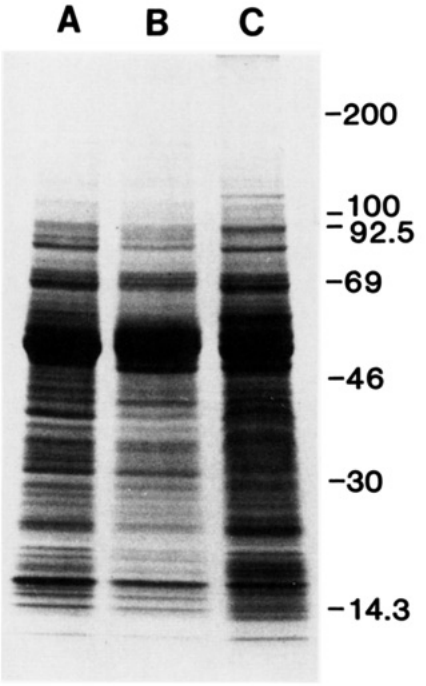

FIGURE 1: Coomassie Brilliant Blue stained SDS–PAGE gel of reduced samples of whole sonicates of different life cycle stages of *T. congolense*. Trypanosomes were prepared as given under Materials and Methods. Lane A, bloodstream forms. Lane B, metacyclic forms. Lane C, procyclic forms.

Western blots of sonicates of various life cycle stages of *T. congolense* IL 3000 were probed with RxCRD. All experiments were done with the same batch of affinity-purified IgG. Different batches of RxCRD, prepared in the same fashion but using unrelated VSGs, gave essentially the same results as described below, and similar results were also observed with bloodstream and metacyclic forms of *T. vivax*, but have not yet been studied so extensively (W. R. Fish and I. D. Gumm, unpublished observations). As expected, procyclics showed no CRD. Reduced samples of bloodstream forms of IL 3000 showed a single band (M_r 55.3 kDa). Nonreduced samples bound less RxCRD, and this band had a slightly lower M_r than that of a reduced sample (Figure 2). In contrast, reduced samples of metacyclics exhibited VSGs with the expected heterogeneity; five bands of varying intensity were observed (49.4–56.8 kDa). RxCRD binding was not altered on blots of a denatured, reduced, and alkylated sonicate of metacyclics. The nonreduced samples of metacyclic traypanosomes showed little or no evidence of RxCRD binding (Figure 2), a phenomenon that was observed in all subsequent experiments. However, the bands observed had a slightly lower apparent M_r than that in reduced samples. The higher apparent M_r and increased binding of RxCRD have been attributed to 1,2-DMG cleavage in the conversion of mfVSG to sVSG (Cardoso de Almeida & Turner, 1983), but decreased mobility of membrane-bound proteins in SDS–PAGE gels can occur upon reduction of disulfide bonds [for references, see Malbon et al. (1987)] and has been shown for at least one other GPI-containing protein (Pierres & Barbet, 1986). Therefore, differences in the M_r of VSG may not be unequivocally attributed to removal of 1,2-DMG.

RxCRD binding to VSGs in reduced samples of the pellet and supernatant of sonicated samples of metacyclic forms of *T. congolense* (Figure 3A,B) differed from hypotonic lysis/freeze–thaw preparations. After hypotonic lysis/freeze–thawing, most of the VSGs that bound RxCRD were found in the supernatant. The pellet from hypotonically lysed metacyclics also showed some RxCRD binding, but the reaction was less than that in the supernatant from the same preparation in the presence or absence of DTT. In contrast, both fractions of sonicated samples showed a similar M_r profile of RxCRD binding, although that obtained from the pellet was much stronger and displayed an overall slight reduction in M_r (Figure 3A,B).

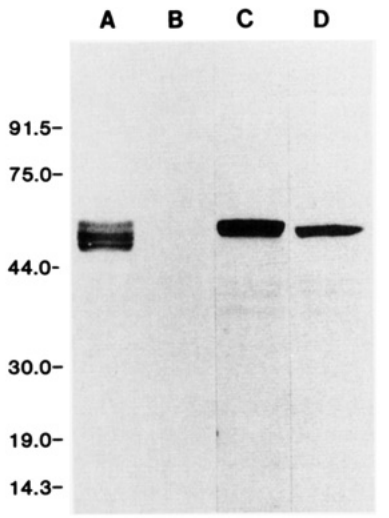

FIGURE 2: Determination of RxCRD binding on Western blots of whole sonicates of metacyclic and bloodstream form *T. congolense*. RxCRD binding was detected as described under Materials and Methods. Lane A, metacyclic forms, reduced. Lane B, metacyclic forms, nonreduced. Lane C, bloodstream forms, reduced. Lane D, bloodstream forms, nonreduced.

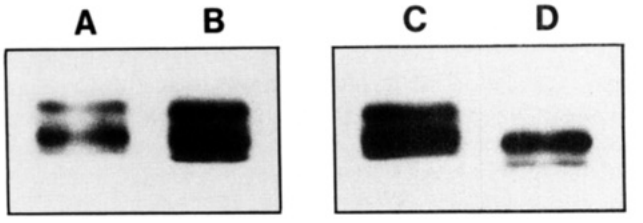

FIGURE 3: RxCRD binding on Western blots of soluble (A) and membrane (B) fractions *or* previously reduced (C) and nonreduced (D) samples of whole sonicates of *T. congolense* metacyclic forms. Samples C and D were subjected to in situ reduction (in the SDS gel) after electrophoresis while samples A and B were already reduced and were not so treated.

An SDS–PAGE gel, which contained both reduced and nonreduced metacyclic sonicates, was exposed to 100 mM DTT for 1 h prior to Western blotting. Some, but not all, bands of the initially nonreduced sample reacted positively with RxCRD and displayed a lower M_r than their initially reduced counterparts (Figure 3C,D). [In this experiment, GPI-PLC interaction with VSG is prevented unless the *T. congolense* enzyme is active in the presence of SDS, and the affected enzyme has multiple M_r's, coincident with respective VSGs. This, however, is inconsistent with our knowledge of trypanosome GPI-PLC (Cardoso de Almeida & Turner, 1983; Bulow & Overath, 1986).] In contrast, DTT treatment of nitrocellulose blots of the same samples, immediately prior to processing, failed to result in RxCRD binding. This suggests that VSG structure on blots, insofar as concerns RxCRD binding, is not altered by in situ reduction. This is not so if nonreduced proteins are reduced within the polyacrylamide gel. Urea treatment, which increases subsequent antibody binding on blots (Erickson et al., 1982), might promote renaturation and/or disulfide bond reformation and yet does not alter RxCRD binding to nonreduced VSGs on Western blots.

Finally, since alkylation of sonicates does not alter RxCRD binding on subsequent Western blots, it is unlikely that disulfide bonds re-form on Western blots.

T. congolense metacyclics were also treated before and after sonication with reducing agents, *p*-(chloromercuri)benzenesulfonate (pCMBS), $ZnCl_2$ (Zn^{2+}), and Zwittergent 3-14 (ZW-314), all reported to affect the GPI-PLC. Less RxCRD was bound at the lower concentrations of DTT (<10 mM) and 2-mercaptoethanol and dithioerythritol substituted for DTT. Sonication in the presence of pCMBS (10 mM), Zn^{2+} (5 mM), or ZW-314 (1% w/v) completely eliminated the binding of RxCRD by the subsequent application of DTT. However, addition of pCMBS, Zn^{2+}, or ZW-314 immediately after sonication allowed RxCRD binding in reduced samples equal to a nontreated, reduced sonicate. When these samples were assayed for GPI-PLC activity, the inhibition of 1,2-DMG release was virtually complete for each inhibitor, whether added before or after sonication, or even in the presence of a 10–20-fold molar excess of DTT. The sole exceptions to this were samples in which pCMBS was present. In the latter case, subsequent addition of excess DTT allowed a recovery of approximately 10% of the original activity. Sonication does not destroy or inhibit the GPI-PLC because the sonicate possessed more activity than a freeze–thaw lysate, and so this activity per se is not relevant to an increase in binding of RxCRD under the conditions used.

A comparison (Figure 4A) of reduced and nonreduced samples of sonicated, [^{3}H]myristate-labeled *T. congolense* metacyclics shows (1) both samples contain equivalent amounts of [^{3}H]myristate label, (2) DTT treatment significantly increases RxCRD binding, and (3) DTT treatment causes an increase in M_r (the reduced sample has no radioactivity below 46 kDa while the nonreduced sample shows significant [^{3}H]myristate label below 46 kDa). To more closely discern the juxtaposition of [^{3}H]myristate and RxCRD binding in reduced samples, a blot of sonicated, [^{3}H]myristate-labeled metacyclics was probed for RxCRD binding using [^{35}S]-streptavidin as a final probe. Figure 4B clearly shows that the two labels are coincident and that no [^{3}H]myristate occurs below 46 kDa. The above results show that, while significant RxCRD binding is displayed, these VSGs still retain the 1,2-DMG moiety (cf. mfVSG) and show an increase in M_r, previously associated with removal of the 1,2-DMG moiety (cf. the mfVSG to sVSG transition; Cardoso de Almeida & Turner, 1983). The shift in M_r that we observe is more likely due to structural changes induced by disulfide bond reduction than to the removal of 1,2-DMG. Thus, a proposal that there is a direct relationship between the extent of RxCRD binding and the presence, or absence, of 1,2-DMG at the C-terminus of VSG may not always be valid (Turner, 1985; Turner et al., 1985).

Since disulfide bond reduction dramatically increased RxCRD binding to VSG, a more detailed examination of the process was warranted. Samples of live *T. congolense* metacyclics solubilized in different detergents and then immediately exposed to hot sample buffer showed no binding of RxCRD irrespective of the detergent used, or the presence or absence of DTT. However, aliquots of these samples, which were allowed to incubate at room temperature (±DTT) prior to heating in sample buffer, showed qualitative and quantitative differences in RxCRD binding (Figure 5). Samples of trypanosomes lysed and incubated in SDS (not shown), sodium deoxycholate, tetradecyltrimethylammonium bromide, and ZW-314 showed no RxCRD binding under any of the incubation conditions. Samples lysed and incubated in CHAPS,

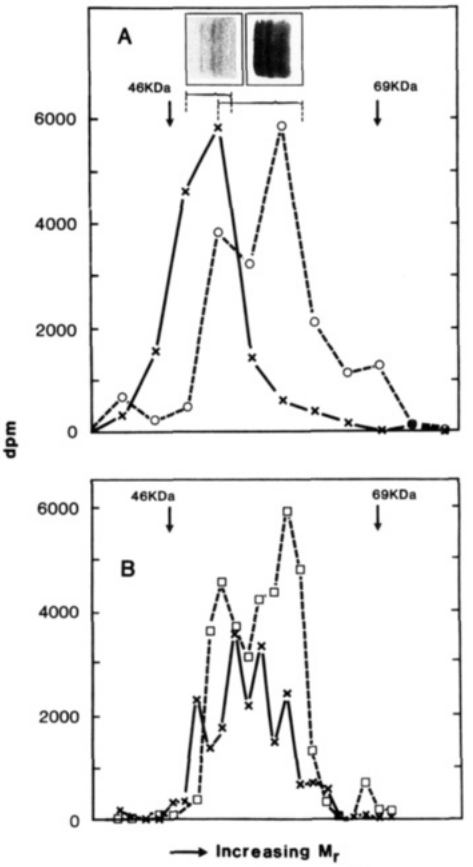

FIGURE 4: Coincidence of RxCRD binding to [^{3}H]myristate-labeled VSGs of metacyclic forms of *T. congolense*. Trypanosomes were labeled with [^{3}H]myristate and sonicated, and RxCRD binding was determined as described under Materials and Methods. (A) After autoradiography, to localize labeled VSGs, the blot was processed for detection of RxCRD binding using SBHRP. The region containing RxCRD was cut into 2.5-mm horizontal slices and counted for ^{3}H by LSC. (×) Nonreduced; (○) reduced. The detected RxCRD binding of each sample is shown above each curve, and the area occupied by each is indicated by brackets. (B) A reduced sample of the sonicate from (A) (above) was blotted and RxCRD binding determined by using [^{35}S]streptavidin as a final probe. The region of VSGs was determined as in (A), and 1-mm horizontal slices were cut. (×) ^{3}H (VSGs); (□) ^{35}S (CRD).

CHAPSO, *n*-octyl glucoside, and Triton X-100 in the presence of DTT bound most RxCRD but when lysed and incubated in the absence of DTT showed little, or no, binding. If DTT was added to the latter samples, after heating in sample buffer, binding of RxCRD was enhanced, but not to the level seen in those containing DTT upon detergent lysis. In contrast to the above observations, when the above detergents were added to sonicated samples (either reduced or nonreduced), the amount of RxCRD bound was identical at all time points (up to 1 h) with that seen with control sonicates lacking detergents. Finally, there is no obvious relationship between the binding of RxCRD and the ionic character or chemical structure of the detergents tested. In all, these results suggest that there is a complex pathway leading to the exposure of the RxCRD binding domain(s), which can be differentially influenced by detergents and DTT.

Discussion

We have studied the immunochemical reactivity of the CRD of the VSGs of metacyclic forms of *Trypanosoma congolense*. Differential binding of RxCRD could be observed depending upon the method of lysate preparation, the addition of a variety of chemicals, and the order of addition of these reagents. We also found that the 1,2-DMG moiety can be present coinci-

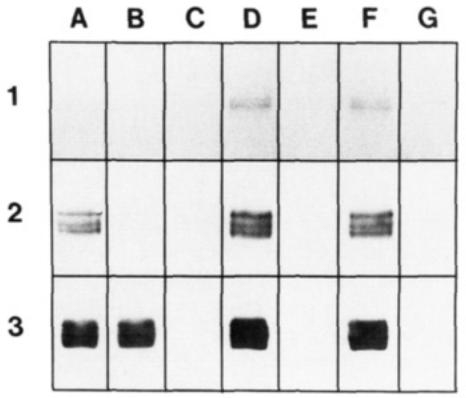

FIGURE 5: Effect of incubation upon RxCRD binding, following direct detergent lysis of live *T. congolense* metacyclics under reducing and nonreducing conditions. Trypanosomes were directly lysed in detergents (±100 mM DTT) and incubated at room temperature. Samples were taken at 0, 5, 15, 30, and 60 min and directly pipetted into boiling sample buffer (±DTT). Samples were Western-blotted, and RxCRD binding was determined. RxCRD binding increased over time, and only 60-min samples are shown. Detergents: (A) CHAPS; (B) CHAPSO; (C) sodium deoxycholate; (D) *n*-octyl glucoside; (E) tetradecyltrimethylammonium bromide; (F) Triton X-100; (G) ZW-314; SDS not shown. Row 1, samples lysed and incubated in the absence of DTT. Row 2, as in row 1 except DTT added prior to SDS–PAGE. Row 3, samples lysed and incubated in the presence of DTT.

dentally with immunochemically demonstrable CRD. Thus, there is no a priori reason for 1,2-DMG to be removed in order to expose epitopes involved in RxCRD binding.

The VSGs of metacyclic forms of *T. congolense* have been studied by Ross et al. (1987), but their results differ from ours. They observed qualitatively similar VSG patterns regardless of whether metacyclic or bloodstream forms were compared, and only one band of VSG was evident in autoradiographs of their ^{125}I-surface-labeled metacyclics. However, as expected from the observations of Luckins et al. (1986), we find that metacyclic forms of *T. congolense* have a group of electrophoretically distinct VSG bands while bloodstream forms contain a single, distinct, broad band of VSG. Some of the differences between our results and those of Ross et al. (1987) may be due to their isolation of trypanosomes using non-purine-containing buffers, which can have significant effects upon the metabolic and physical integrity of trypanosomes (Lonsdale-Eccles & Grab, 1987), and/or by their use of insufficient concentrations of *N*-*p*-tosyl-L-lysine chloromethyl ketone, which is only a moderately effective inhibitor of trypanosome proteases (Lonsdale-Eccles & Mpimbaza, 1986).

We also found that *T. congolense* metacyclics subjected to hypotonic/freeze–thaw lysis [as used by Ross et al. (1987)] displayed results exactly the opposite of those seen with sonicates. In a hypotonic/freeze–thaw lysate, the supernatant, rather than the pellet, showed greater binding of RxCRD to VSGs. In contrast, in samples of sonicated metacyclics, most RxCRD binding VSGs resided in the pellet, and these VSG bands were of a slightly lower apparent M_r than those found in the soluble fraction of the same preparation. Thus, they behave like mfVSG. Consequently, under appropriate conditions of parasite lysate preparation, the membrane fraction, which behaves like and should contain predominantly mfVSG, contains molecules that readily bind RxCRD.

Physical disruption of trypanosomes (e.g., hypotonic lysis and/or freeze–thawing) usually results in the uniform production of sVSG and immunoreactivity of the CRD. Execution of the same procedures in the presence of pCMBS, Zn^{2+}, or ZW-314 preserves mfVSG and greatly reduces the binding of RxCRD, presumably due to the inhibition of GPI-PLC. The GPI-PLCs of *T. b. brucei* bloodstream forms and *T. congolense* metacyclics have also both been reported to be heat labile (Ward et al., 1987; Ross et al., 1987). However, in sonicated material, we find that RxCRD binding is greatly increased merely by the addition of a disulfide reducing agent, despite the sonicate having been heated at 100 °C in SDS–PAGE sample buffer or the addition pCMBS, Zn^{2+}, or ZW-314 immediately after sonication. We also find that sonicates prepared with the latter three reagents show little or no GPI-PLC activity. Therefore, the current proposals (Bulow & Overath, 1985; Cardoso de Almeida et al., 1984; Gurnett et al., 1986) that these reagents block the production of sVSG from mfVSG, by inhibiting the GPI-PLC (resulting in decreased RxCRD binding), must be viewed with some caution. Alternative explanations are possible that are distinct from, but not exclusive of, the inhibition of GPI-PLC. For example, pCMBS and Zn^{2+} have been shown to cause profound concentration-dependent disturbances in the membrane/cytoskeleton of erythrocytes [see Kunimoto et al. (1987) and other references cited therein] and to affect the formation of tubulin polymers (Wallin et al., 1977). These effects were due to processes involving disulfide bonds, not phospholipases.

The mfVSG to sVSG transition and the concomitant increase in both M_r and RxCRD binding are time-dependent events (Cardoso de Almeida & Turner, 1983) that are inhibitable by a variety of external effectors (see above). However, we find that neither these effectors nor lengthy incubation (with or without noninhibitory detergents and/or DTT) influences the extent of binding of RxCRD to the VSGs in sonicates of *T. congolense* metacyclics. We therefore propose that sonication may result in the generation of a stable intermediate form of VSG in which exposure of epitopes for RxCRD binding only requires reduction of disulfide bonds. This resembles the reduction-dependent binding of RxCRD to the temperature-specific surface antigens of *Paramecium primaurelia* (Capdeville et al., 1987) and further suggests that disulfide reduction may be affecting the VSG (as a substrate) rather than activating the GPI-PLC (Hereld et al., 1986).

In addition to the present studies, the relationship between cleavage of lipid and other carboxy-terminal moieties of GPI-containing proteins, RxCRD binding, and production of soluble forms of these proteins has been extensively investigated. Two different human serum enzymes have been described (one cleaves myristic acid from mfVSG and the other dimyristoylphosphatidic acid from decay accelerating factor) that generate the soluble form of the protein but do not result in an increase in RxCRD binding (Cardoso de Almedia et al., 1988; Davitz at al., 1987a,b). Romero et al. (1988) report that the release of the GPI-anchored alkaline phosphatase from the membrane of myocytes is independent of 1,2-DMG release. The action of the GPI-PLC, in the cases of decay accelerating factor (Shak et al., 1988) and VSG (Ferguson et al., 1985), results in a carboxy-terminal inositol cyclic 1,2-phosphate which may in part be responsible for the binding of RxCRD. Others describe the necessity to cleave a carboxy-terminal phosphate bond in mfVSGs in order to release sVSG and/or increase RxCRD binding (Jackson & Voorheis, 1985; Schmitz et al., 1987). Galactose has also been found to influence RxCRD binding (Menon et al., 1988). Recently, a detailed study (Zamze et al., 1988) reported that polyclonal RxCRDs are directed against three overlapping epitopes on VSG, namely, inositol cyclic 1,2-phosphate, glucosamine, and the galactose branch of an oligosaccharide chain. Although the latter study confirms some of the observations of others, the

use of a raw antiserum, in both ELISA and Western blots, is unfortunate. In addition, the two VSGs used, one for raising the antiserum and the other as an assay antigen, were both derived from trypanosome variants arising from the same initial isolate and, therefore, may display cross-reactivity that is unrelated to the CRD (Barbet et al., 1989). In the present work, this possibility is minimized by using affinity-purified RxCRD against a different species and a different life cycle stage from those used to obtain the IgG.

Our observation that the removal of 1,2-DMG from metacyclic VSGs is not prerequisite for RxCRD binding is difficult to reconcile with the suggestion that the action of GPI-PLC upon mfVSG is necessary for exposure of CRD epitopes to antibody binding (Turner, 1985; Turner et al., 1985). Rather, our results suggest that, at the chemical level, the exposure of CRD epitopes is a complex, multistep process involving two or more structural intermediates. This is supported by the differential manner in which binding of RxCRD is influenced by the method of lysis, time, detergents, external effectors, and the presence or absence of DTT. Although other scenarios are possible, we propose that sonication either directly or indirectly causes structural changes in the VSG. Subsequently, reduction of disulfide bonds suffices to complete the opening of the VSG structure for RxCRD binding to CRD epitopes which are cryptic in the intact and folded molecule. The initial change in VSG folding may occur at a "hinge" region (Gomes et al., 1986) which could connect domains of both *T. brucei* and *T. congolense* VSGs (Clarke et al., 1988; Cohen et al., 1984; Strickler et al., 1987). Such regions of a molecule are often protease sensitive (Fontana et al., 1986), and protease-sensitive sites are known in VSGs of bloodstream forms of *T. b. brucei* and *T. congolense* (Johnson & Cross, 1979; Reinwald et al., 1987).

An inherent conclusion of our studies is that disulfide bonds are an essential component in maintaining the secondary and tertiary structure in the CRD region of the VSG of *T. congolense*. Cysteine residues in the C-terminus of *T. b. brucei* VSGs are relatively conserved (Rice-Ficht et al., 1981), and disulfide bonds are important in the maintenance of a tight structural configuration in this region of the protein (Allen & Gurnett, 1983). Curiously, Strickler et al. (1987) observed that there was an absence of cysteine residues in the carboxy-terminal one-fourth of two VSGs of bloodstream forms of *T. congolense* and that the majority of conserved cysteines occurred in the middle of these molecules. Nevertheless, the obvious complexity of the process leading to increased immunochemical reactivity of CRD epitopes warrants a closer examination even if it is only to determine whether our findings have relevance to the in vivo situation.

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Detection of Intermediates in the Unfolding Transition of Phosphoglycerate Kinase Using Limited Proteolysis†

Jean-Michel Betton, Michel Desmadril, and Jeannine M. Yon*

Laboratoire d'Enzymologie Physico-chimique et Moléculaire, Groupe de Recherche du Centre National de la Recherche Scientifique associé à l'Université de Paris-Sud, 91405 Orsay, France

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ABSTRACT: The accessibility of peptide bonds to cleavage by *Staphylococcus aureus* V8 protease bound on a Sepharose matrix was used as a conformational probe in the study of the unfolding–folding transition of phosphoglycerate kinase induced by guanidine hydrochloride. It was shown that the protein is resistant to proteolysis below a denaturant concentration of 0.4 M. The transition curve, determined by susceptibility toward proteolysis, was similar to that obtained following the enzyme activity [Betton et al. (1984) *Biochemistry 23*, 6654–6661]. Proteolysis under conditions where the folding intermediates are more populated, i.e., 0.7 M Gdn·HCl, gave two major fragments of M_r 25K and 11K, respectively. The 25K polypeptide fragment was identified as the carboxy-terminal domain. Its conformation was similar to that of a folding intermediate trapped at a critical concentration of denaturant, and in this form, it was not able to bind nucleotide substrates [Mitraki et al. (1987) *Eur. J. Biochem. 163*, 29–34]. From the present data and those previously reported, we concluded that the intermediate detected on the folding pathway of phosphoglycerate kinase has a partially folded carboxy-terminal domain and an unfolded amino-terminal domain.

In relatively large globular proteins, the polypeptide chain folds in distinct structural regions called domains (Edelman, 1970). These domains have been considered as structural, genetic, and functional units (Janin, 1979; Rossmann & Argos, 1981). Several methods for establishing the presence of domains in proteins from X-ray structures have been developed (Ooi & Nishikawa, 1973; Rossmann & Liljas, 1974; Rose, 1979; Wodak & Janin, 1980). It was suggested that the domains in such proteins result from gene fusion or insertion during evolution. Correlation among exons in genes and protein structural units, which permits the production of new proteins by bringing together the corresponding segments, has been discussed (Gilbert, 1978; Blake, 1978; Gô, 1981).

Wetlaufer (1973) proposed that the early stages of the protein folding process could occur independently in each of these domains. According to this point of view, particular segments of an unfolded polypeptide chain first refold to form individual domains, which then associate and interact to give the final tertiary structure, as do subunits in oligomeric proteins. The folding of polypeptide fragments corresponding to structural domains has been reviewed by Wetlaufer (1981), Ghélis and Yon (1982), and Jaenicke (1987). The major implication of this model is that the portions of the protein corresponding to domains in the whole protein are expected to fold into a nativelike structure independently from the rest of the polypeptide chain. Moreover, as emphasized by Jaenicke (1987), folding units as domains "may be obligatory inter-

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