ATP/ADP Binding to a Novel Nucleotide Binding Domain of the Reticulocyte-binding Protein Py235 of Plasmodium yoelii*

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The mechanism by which a malaria merozoite recognizes a suitable host cell is mediated by a cascade of receptor-ligand interactions. In addition to the availability of the appropriate receptors, intracellular ATP plays an important role in determining whether erythrocytes are suitable for merozoite invasion. Recent work has shown that ATP secreted from erythrocytes signals a number of cellular processes. To determine whether ATP signaling might be involved in merozoite invasion, we investigated whether known plasmodium invasion proteins contain nucleotide binding motifs. Domain mapping identified a putative nucleotide binding region within all members of the reticulocyte-binding protein homologue (RBL) family analyzed. A representative domain, termed here nucleotide binding domain 94 (NBD94), was expressed and demonstrated to specifically bind to ATP. Nucleotide affinities of NBD94 were determined by fluorescence correlation spectroscopy, where an increase in the binding of ATP is observed compared with ADP. ATP binding was reduced by the known F1F0-ATP synthase inhibitor 7-chloro-4-nitrobenzo-2-oxa-1,3-diazole. Fluorescence quenching and circular dichroism spectroscopy of NBD94 after binding of different nucleotides provide evidence for structural changes in this protein. Our data suggest that different structural changes induced by ATP/ADP binding to RBL could play an important role during the invasion process.

Malaria is caused by unicellular protozoan parasites and is considered to be one of the most important infectious diseases still affecting mankind today. The complex life cycle of the parasite is characterized by three invasive forms: the sporozoite and merozoite that invade hepatocytes and erythrocytes in the vertebrate host, respectively, and the ookinetes inside the insect vector that penetrates the mosquito midgut epithelium (1–5). In the case of merozoites, specific organelles (rhoptries, micronemes, and dense granules) at the apical end of the parasite can invade (20). Studies carried out on the RBLs of Plasmodium falciparum (PfRH1, -2a, -2b, -3, and -4) (10, 14–16, 23, 24) have provided additional evidence that different RBL members interact with different receptors on the erythrocyte and that these interactions are crucial for invasion. Previoulsy work in Plasmodium vivax has indicated that RBLs may have an initial sensing role preceding and possibly enabling the subsequent interaction of the erythrocyte binding ligand member with its corresponding receptor (25). In a more recent study the erythrocyte binding region of PfRH1 and PfRH4 has been identified (26, 71), showing that only a relatively small region of these proteins is actually involved in receptor binding. Recognition of the appropriate receptor on the erythrocyte by RBL is expected to lead to downstream effects that ultimately enable the parasite to continue the invasion process. Although all the studies done on RBLs in the different Plasmodium species have highlighted the importance of this protein family, they have given us little insight in terms of the underlying biochemistry that allows RBL to mediate its function. This problem is due in part to the variation of the host cell (6). Recognition of specific erythrocyte receptors by the merozoite is mediated by at least two gene families, the reticulocyte-binding protein homologues (RBL) and the erythrocyte binding ligands (7, 8). Like the erythrocyte binding ligands, the RBLs are also found in varying numbers in all plasmodium species with each member believed to play a role in recognizing a different receptor (7–16). In general, the members of RBL are large transmembrane proteins with molecular masses above 200 kDa that get proteolytically cleaved during the invasion process (14, 17). In Plasmodium yoelii, members of the RBL termed Py235 (P. yoelii 235-kDa rhoptry protein) have been shown to be potential virulence factors that enable the parasite to invade a wider range of host erythrocytes (18–20). In addition, Py235 is also involved in clonal phenotypic variation of merozoites (21) enabling the parasite to evade immune responses and adapt to changes in the host environment during the invasion step (22). Recently it has been demonstrated that variation in the amount of Py235 expressed in merozoites defines the host cell repertoire that a parasite can invade (20). Studies carried out on the RBLs of Plasmodium falciparum (PfRH1, -2a, -2b, -3, and -4) (10, 14–16, 23, 24) have provided additional evidence that different RBL members interact with different receptors on the erythrocyte and that these interactions are crucial for invasion. Previoulsy work in Plasmodium vivax has indicated that RBLs may have an initial sensing role preceding and possibly enabling the subsequent interaction of the erythrocyte binding ligand member with its corresponding receptor (25). In a more recent study the erythrocyte binding region of PfRH1 and PfRH4 has been identified (26, 71), showing that only a relatively small region of these proteins is actually involved in receptor binding. Recognition of the appropriate receptor on the erythrocyte by RBL is expected to lead to downstream effects that ultimately enable the parasite to continue the invasion process. Although all the studies done on RBLs in the different Plasmodium species have highlighted the importance of this protein family, they have given us little insight in terms of the underlying biochemistry that allows RBL to mediate its function. This problem is due in part to the

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4 The abbreviations used are: RBL, reticulocyte-binding protein homologue; NBD, nucleotide binding domain; SMART, Simple Modular Architecture Research Tool; PSI BLAST, Position-Specific Iterated Basic Local Alignment Search Tool; DTT, dithiothreitol; AMP-PNP, adenosine 5′-(β,γ-imino)triphosphate; MALDI-TOF, matrix-assisted laser desorption-ionization/time-of-flight; FCS, fluorescence correlation spectroscopy; LSM, laser scanning microscopy; EDA-ATP, 2′,3′-O-(2-aminoethylcarbamoyl)adenosine 5′-triphosphate; 8-N2, 3′-biotinyl-ATP, 8-azido-3′-biotinyl adenosine 5′-triphosphate; DBS, dichroic beam splitter; EF, emission filter; NBD-CI, 7-chloro-4-nitrobenzo-2-oxa-1,3-diazole; P-loop, phosphate-binding loop.

* This work was supported by Agency for Science, Technology and Research (A*STAR) Biomedical Research Council Grant 06/1/22/19/456 and Academic Research Council Grant MLCO3/03. The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked “advertisement” in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.
fact that the overall sequence conservation between the different members of the RBL family is very low and that no conserved domains have been identified so far that would indicate important functional regions within this class of proteins.

Although the availability of erythrocyte surface proteins is a key step in the recognition and subsequent invasion by the merozoite it is not clear what and whether other factors are important for the invasion process. ATP released by erythrocytes under normal physiological conditions has recently been shown to serve as a signaling molecule regulating vascular tone (27–29), and ATP receptors have now been implicated in a number of signaling pathways (30, 31). Mechanical deformation of erythrocytes as encountered in capillaries forming the microvasculature leads to increased ATP release (29). Importantly intracellular ATP is a requirement for merozoite invasion (32–35); erythrocytes that have been depleted of ATP are refractory to invasion. These findings imply that merozoites can sense the intracellular ATP level of the erythrocyte. To explore this further, we investigated merozoite proteins that are thought to play a role in host cell recognition for the presence of putative nucleotide binding domains.

Here a nucleotide binding domain that is highly conserved among the RBLs is described. We report for the first time the property of ATP/ADP binding of this expressed domain, termed nucleotide binding domain 94 (NBD94), independently observed by photoaffinity labeling and fluorescence correlation spectroscopy. Decreased tyrosine fluorescence and changes of circular dichroism spectra in the presence and absence of nucleotides are discussed in light of structural alterations in NBD94 and its possible role in serving as an ATP/ADP sensor during the invasion process.

**EXPERIMENTAL PROCEDURES**

*Biochemicals—P. yoelii* YM frozen stabilates in liquid nitrogen were obtained from the National Institute of Medical Research. *Pfu* DNA polymerase and restriction enzymes were purchased from New England Biolabs. Ni²⁺-Sepharose™ High Performance chromatography resin was obtained from GE Healthcare. Chemicals for gel electrophoresis were received from Bio-Rad. All other chemicals were at least of analytical grade and purchased from Sigma-Aldrich, Amersham Biosciences, BIOMOL (Hamburg, Germany), and Merck.

*Bioinformatics—*The nucleotide sequence data for the various Py235 members were obtained from PlasmoDB, *Plasmodium* genome resource, Version 4.4. PlasmDB was also used to obtain nucleotide sequences of other *Plasmodium* erythrocyte- and reticulocyte-binding proteins. The protein sequences were then predicted based on the gene sequences obtained using PlasmDB. Manipulation of nucleotide and protein sequences was carried out using Vector NTI and The Sequence Manipulation Suite. Alignment of these sequences was done using ClustalW (European Molecular Biology Laboratory-European Bioinformatics Institute). The protein sequences were further analyzed using Normal SMART (Simple Modular Architecture Research Tool) program (36, 37) and PSI BLAST (Position-Specific Iterated Basic Local Alignment Search Tool) to search for a conserved domain/motif.

**Nucleotide Binding Traits of the NBD 94 Domain**

*Parasite Strains and Genomic DNA Extraction—*Frozen stabilates of *P. yoelii* YM were separately taken up in 0.9% saline with glucose and injected intraperitoneally into 4–6-week-old BALB/c mice bred under specific pathogen-free conditions as described previously (38). Genomic DNA was extracted from infected blood using the Easy DNA kit (Invitrogen) according to the manufacturer’s protocol. Isolated DNA was stored at −20 °C.

*Cloning of the NBD94 Domain of PY01365 from P. yoelii—*The predicted nucleotide binding domain of PY01365 (hereafter termed NBD94) was amplified using ~100 ng of genomic YM DNA as a template and oligonucleotide primers 5’-GTGAGTCCATGTTATCTGACAAAAATGAATATG-3’ (forward primer) and 5’-AAATTACGAGCTTCATTATTTTACCATC-TACAG-3’ (reverse primer). These primers were designed specially to include Ncol and SacI restriction sites (underlined), respectively. After digestion with Ncol and SacI, the PCR products were ligated into pET9d-His6 vector (39), and the recombinant was then transformed into Escherichia coli DH5α competent cells. Successful transformants were screened and verified by DNA sequencing. The correctly sequenced recombinant was then transformed into Rosetta-gami™(DE3) cells (Novagen) and grown on Luria-Bertani (LB) agar plates containing 30 μg/ml kanamycin and 34 μg/ml chloramphenicol.

*Expression and Purification of NBD94—*To express His6-NBD94, liquid cultures were incubated with agitation in LB medium containing 30 μg/ml kanamycin and 34 μg/ml chloramphenicol at 37 °C for about 3 h at 200 rpm until an A₆₀₀ of 0.6–0.8 was reached. To induce the production of His6-NBD94, the cultures were supplemented with isopropyl β-d-thiogalactopyranoside to a final concentration of 1 mM and further incubated for another 3 h at 37 °C. The cells were then harvested at 8,000 × g for 12 min at 4 °C. These cells were then subsequently lysed on ice by sonication (Sonopuls HD2200, Bandelin, KE76, 20%) for 3 × 1 min in buffer A (50 mM Tris/ HCl, pH 7.5, 250 mM NaCl, 4 mM PefablocSC (BIOMOL), 0.7 mM DTT). The lysate was cleared by centrifugation at 10,000 × g for 35 min at 6 °C, and the supernatant was filtered through a 0.45-μm-pore size filter and added to the Ni-Sepharose™ resin. Binding of the His-tagged proteins to the matrix was carried out for 90 min at 4 °C, and proteins were eluted with an imidazole gradient (0–250 mM) in buffer A. Fractions containing His₆-NBD94 were identified by SDS-PAGE (40), pooled, and applied on an ion exchanger (DEAE column HiPrep™ 16/10, GE Healthcare), which was equilibrated in 50 mM Tris/ HCl, pH 7.5, 0.7 mM DTT, 1 mM phenylmethylsulfonyl fluoride.

The protein was eluted using a linear gradient with buffer B (50 mM Tris/HCl, pH 7.5, 1 mM NaCl, 0.7 mM DTT, 1 mM phenylmethylsulfonyl fluoride) at 3 ml/min. Following elution, concentration was carried out using Centricon YM-50 (50-kDa cutoff) spin concentrators (Millipore), and the purity of the eluted protein was assessed by SDS-PAGE. The SDS gels were stained with Coomassie Brilliant Blue G-250. Western blot analyses using mouse anti-penta-His (Qiagen) were performed as described previously (41). ATPase activity was measured according to Heinonen and Lahti (42) and Chan et al. (43) in which the spectrophotometric quantification of the phosphomolydbdate-malachite green complex is used.
Nucleotide Binding Traits of the NBD 94 Domain

Cloning, Production, and Purification of NBD94-(1–550)—A truncated version of the full-length NBD of PY01365 (hereafter termed NBD94-(1–550)) was cloned, expressed, and purified in a manner similar to that above with the following changes. The reverse primer used for amplification is 5′-AATAC-GAGGCCTTAAATCTTGTAATCCTTCTAA-3′. The recombinant plasmid was eventually transformed in BL21 (DE3) cells (Novagen) and grown on LB agar plates containing 30 μg/ml kanamycin. NBD94-(1–550) was purified using Ni²⁺-Sepharose followed by an ion exchanger as described for full-length NBD94 (see above). It was further purified via size exclusion chromatography using Superdex™ 75 10/300 GL (Amersham Biosciences) in 50 mM Tris/HCl, pH 7.5, 200 mM NaCl, 1 mM phenylmethylsulfonyl fluoride, 1 mM DTT.

Circular Dichroism Spectroscopy—Measurements of steady state CD spectra were carried out in the far-UV light (185–260 nm) using a Chirascan spectropolarimeter (Applied Photophysics). The CD spectroscopy measurements of NBD94 and NBD94-(1–550) in Tris/HCl, pH 7.5, 160 mM NaCl, 0.7 mM DTT and Tris/HCl, pH 7.5, 200 mM NaCl, 1 mM DTT, respectively, were done using a 60-μl quartz cell (Helma) at 18 °C with 1-nm step points. Independent of the presence or absence of nucleotides all samples were incubated for 15 min with rocking at 4 °C before the start of each measurement. The spectrum for the buffer was subtracted from the spectrum of the protein. The ellipticity values were calculated by the average of triple determinations for each sample with a bandwidth of 1 nm from 185 to 260 nm (1-nm step points). The CD data were converted to mean residue ellipticity (θ) in units of degrees × dmol⁻¹ × amino acid⁻¹ using the software Chirascan version 1.2.1 (Applied Photophysics). Base line-corrected spectra were used as input for computer methods to obtain predictions of secondary structure. To analyze the CD spectrum the following algorithms were used: Varselec (44), Selcons (45), Contin (46), K2D (47), and all methods as incorporated into the program Dicroiter (48) and NeuralNet (49).

Photoaffinity Labeling—Photoaffinity labeling was done as described by Schäfer et al. (50). Protein was incubated with 5 × 10⁻⁴ M 8-N3-biotinyl-ATP and 5 × 10⁻⁴ M MgCl₂ in 50 mM Tris/HCl, pH 7.5, 250 mM NaCl, 0.7 mM DTT for 5 min on ice in the dark followed by irradiation with an ultraviolet lamp at 366 nm for 30 min on ice. The samples were applied on SDS-PAGE after which a Western immunoblot was carried out to identify the labeled (biotinylated) protein using streptavidin/peroxidase conjugate. The competition assay was also done concurrently with nucleotide analogues (ATP, ADP, and AMP-PNP).

In-gel Tryptic Protein Digestion and Mass Spectrometric Analysis—The 8-N3-biotinyl-ATP-labeled and unlabeled bands were cut out from the SDS-PAGE gel and destained with a solution of 25 mM ammonium bicarbonate and 50% acetonitrile for 12 h. The gel band was cut into small pieces of 1 mm³, washed three times with acetonitrile, dried for 30 min in a speed vacuum concentrator, and digested with trypsin overnight (51, 52). For matrix-assisted laser desorption-ionization/time-of-flight (MALDI-TOF) spectrometry, aliquots of digested samples were applied to a target disk and allowed to air dry. Subsequently matrix solution (1% (w/v) α-cyano-4-hydroxyckcinamic acid in 50% acetonitrile, 0.1% (v/v) trifluoroacetic acid) was applied to the dried sample and again allowed to dry. Peptide mass mapping was performed using an ABI 4800 MALDI-TOF mass spectrometer. For interpretation of the protein fragments, the PEPTIDEMASS (53) program available from ExPAsy was used.

Fluorescence Correlation Spectroscopy (FCS)—FCS was performed on an LSM-FCS system at room temperature (ConfoCor 3, Zeiss, Jena/Germany) using the ATP analogue EDA-ATP ATTO-647N and the ADP analogue EDA-ADP ATTO-TEC (Siegen, Germany). The FCS experiments were performed in 50 mM Tris/HCl, 150 mM NaCl, pH 7.5, with 1 mM DTT.

The 633 nm laser line of a 5-milliwatt HeNe633 laser was focused into the aqueous solution by a water immersion objective (40×/1.2 W Corr UV-VIS-IR, Zeiss). FCS was measured in 15- or 50-μl droplets, which were placed on a gelatin-treated Nunc 8-well chambered cover glass according to Hunke et al. (54). The following filter sets were used: main beam splitter, Hauptfarbteiler, main color splitter 488/543/633; EF1 (emission filter), LP 655; EF, none; dichroic beam splitter (DBS), none; 633 nm, 3% transmission. Out-of-focus fluoroscence was rejected by a 90-μm pinhole in the detection pathway, resulting in a confocal detection volume ~0.25 fl. Fluorescence autocorrelation functions were measured for 30 s each with 10 repetitions. Cyanine 5 solutions in buffer were used as references for the calibration of the ConfoCor 3 system.

To study the effect of 7-chloro-4-nitrobenzo-2-oxa-1,3-diazole (NBD-Cl), protein solution, magnesium chloride, and NBD-Cl were preincubated for 30 min at 4 °C. After addition of EDA-ATP ATTO-647N the solution was incubated for further 10 min. The following filter sets were used: main beam splitter, Hauptfarbteiler, main color splitter 514/633; EF1 (emission filter), LP 655–710R; EF, none; DBS, none; DBS1, plate; DBS3, mirror; 633 nm, 3% transmission. To analyze the autocorrelation functions, a standard model was used for fitting (FCS-LSM software, ConfoCor 3, Zeiss). The calculations were done by the ConfoCor 3 software 4.2, Microsoft Excel 2003, and Origin 7.5.

Tyrosine Fluorescence Spectroscopy—A Varian Cary Eclipse spectrofluorometer was used, and all experiments were carried out at 20 °C. The samples were excited at 277 nm, and the emission was recorded from 295 to 350 nm with excitation and emission band passes set to 5 nm.

Preparation of P. yoelii YM Culture Supernatant—YM parasites were harvested from BALB/c mice, and schizonts were purified by centrifugation on a 50–80% step gradient of Nyco-denz (Sigma). Purified schizonts were placed back into culture containing incomplete RPMI 1640 medium with 25% fetal bovine serum (Invitrogen). Cells were harvested by centrifugation after 16 h. To remove residual nucleotides the supernatant was dialyzed against incomplete RPMI 1640 medium at 4 °C overnight and stored in aliquots at −80 °C for further erythrocyte binding assays.

Erythrocyte Binding Assay Using Untreated and Nucleotide-treated Dialedyzed P. yoelii YM Parasite Culture Supernatant—Erythrocyte binding assays were performed with minor modifications as described previously (13, 55). Briefly 30 μl of dialyzed supernatant were incubated with a final concentration of 3 mM MgATP (ratio of 1:1), 3 mM MgADP (ratio of 1:1), or no nucleotide addition in incomplete RPMI 1640 medium at 4 °C.
for 15 min, respectively, followed by the addition of 100 μl of packed BALB/c mice erythrocytes. The bound protein was eluted, separated by SDS-PAGE on a 6% polyacrylamide gel, and detected by Western blotting using mouse monoclonal antibody 25.77 (18, 19).

RESULTS

Domain Mapping of Merozoite Invasion Proteins Using Bioinformatics Tools—The phosphate-binding loop (P-loop) is one motif commonly found in adenine nucleotide-binding proteins. The consensus sequence for the P-loop motif is normally quoted in the literature as GXXXXGK/TX (56, 57). Some ATP-binding proteins share another less conserved site with unknown function, originally called motif B (57). Although there are proteins known to bind ATP that lack a P-loop motif, like glycolytic kinases, E1/E2-type ATPases, actin, tubulin, and aminoacyl-tRNA synthetases (56), we nevertheless searched for the P-loop patterns using the program SMART in known merozoite invasion proteins. As shown in Table 1, an NBD with an e-value of 2 × 10^{-35} was identified in the case of the Py235 family member PY01365 from P. yoelii. Similar values could be determined for other Py235 members, such as PY01185 and PY00649. It also became evident that the NBD is predicted with a very high level of confidence (1 × 10^{-12}–2 × 10^{-35}) in all Py235 members. Additional confirmation came from another motif prediction program, PSI BLAST, which identified this region as a potential nucleotide binding domain for the Py235 members (data not shown). The same analysis was carried out on the highly diverse homologues of the RBL family in other Plasmodium species as well as a range of other unrelated malaria invasion proteins. An NBD was also predicted with a high degree of confidence level in all members of the PRRH (2 × 10^{-11}–2 × 10^{-29}) and PVRBP (6 × 10^{-22}–6 × 10^{-25}) family. The location of the predicted NBD is similar within members of the RBL of the same species (Table 1). Importantly no NBD was predicted for other types of malaria invasion proteins, such as apical membrane antigen, merozoite surface proteins 1–7, the erythrocyte binding ligand families, Rhoptry-associated proteins 1–3, high molecular weight rhoptry proteins 1 and 3, rhoptry-associated membrane antigen, surface-associated intersperse genes, and apical Sushi protein (data not shown). Only in the case of RhopH2 was a putative NBD identified, although with significantly reduced confidence (1 × 10^{-9}). Walker A and Walker B motifs were identified in the primary amino acid sequence of the predicted NBD of PY01365 (Fig. 1). A glycine-rich region, GTPKGNT, was eluting at 125–200 mM imidazole was collected and used to separate NBD94 from the main contaminating proteins. The identity of the protein was confirmed by mass spectroscopy, NMR and cyclic voltammetry (Fig. 2).

Nucleotide Binding Traits of the NBD 94 Domain

**TABLE 1** SMART program-predicted nucleotide binding domains within the protein sequences of RBLs in *P. yoelii*, *P. falciparum*, and *P. vivax*

| RBL proteins | Nucleotide binding domain (amino acid sequence length) | Position | e values |
|--------------|--------------------------------------------------------|----------|---------|
| Py235        |                                                       |          |         |
| PY05995      | 474                                                   | 405–878  | 1.00e–12|
| PY06018      | 603                                                   | 45–647   | 6.00e–17|
| PY05054      | 661                                                   | 492–1152 | 5.00e–15|
| PY03534      | 808                                                   | 204–1011 | 2.00e–25|
| PY03184      | 658                                                   | 314–971  | 2.00e–20|
| PF03185      | 793                                                   | 611–1403 | 2.00e–35|
| PY01851      | 928                                                   | 602–1259 | 2.00e–29|
| PY04630      | 367                                                   | 666–1032 | 4.00e–15|
| PY00649      | 599                                                   | 746–1344 | 2.00e–31|
| PY02104      | 792                                                   | 653–1444 | 2.00e–22|
| PY07740      | 658                                                   | 319–976  | 9.00e–18|
| PRRHs        |                                                       |          |         |
| PRRH1        | 662                                                   | 1460–2121| 2.00e–16|
| PRRH2a       | 631                                                   | 1300–1930| 2.00e–20|
| PRRH2b       | 661                                                   | 1225–1885| 2.00e–20|
| PRRH3        | 636                                                   | 1238–1873| 7.00e–18|
| PRRH4        | 602                                                   | 201–802  | 2.00e–11|
| PVRRPs       |                                                       |          |         |
| PVRRP-1      | 808                                                   | 975–1782 | 6.00e–22|
| PVRRP-2      | 614                                                   | 602–1215 | 6.00e–25|
confirmed by MALDI-TOF mass spectrometry. The monodispersity of NBD94 was proven by fluorescence correlation spectroscopy (see below). The secondary structure of NBD94 was determined from circular dichroism spectra measured between 185 and 260 nm (Fig. 2). The minima at 222 and 208 nm and the maximum at 192 nm indicate the presence of α-helical structures in the protein. The secondary structure content was calculated to be 65% α-helix and 24% random coiled. This result is consistent with secondary structure predictions based on NBD94 amino acid sequence. The molar ellipticity value at 208 nm is 36390 deg × dmol⁻¹ × cm⁻¹, respectively, in a ratio of 1.0. Because non-interacting helices typically give ratios of around 0.8, whereas interacting helices have ratios close to 1.0 (58), the CD spectrum presented indicates that many of the residues in NBD94 are in strong interaction. An ATPase activity of the NBD94 protein in the presence of MgCl₂ could not be detected when compared with the standard hydrolytic activity of 11–13 μmol of ATP hydrolyzed/mg/ml of the E. coli F₁-ATPase (59).

FIGURE 2. Far-UV CD spectra of NBD94 in the absence (---) and presence of 2 mM MgATP (-----) and MgADP (---), respectively. Inset, an SDS gel shows a sample of the purified NBD94. deg, degrees; aa, amino acid.

Interaction of the Monofunctional Label 8-N₃-3’-Biotinyl-ATP with NBD94—There has been considerable interest recently in the use of photoaffinity analogues to characterize the structure-function relationship of ATPases and nucleotide-binding proteins from different sources (50, 60, 61). Here 8-N₃-3’-biotinyl-ATP, which reacts predominantly with nucleophilic groups (50), was supplemented with nucleotide-depleted NBD94. After irradiation of NBD94 in the presence of Mg²⁺-8-N₃-3’-biotinyl-ATP, the bound photoaffinity label was detected by Western blotting using streptavidin. The results are shown in Fig. 3A, lane 3. To test whether labeling could be protected by the natural substrates MgATP (lane 6) and MgADP (lane 5) or by the non-hydrolyzable analogue Mg²⁺-AMP-PNP (lane 4), these nucleotides were added prior to the labeling procedure. As can be seen, the presence of MgATP and Mg²⁺-AMP-PNP as well as MgADP prevented binding of Mg²⁺-8-N₃-3’-biotinyl-ATP to the protein. Also irradiation of NBD94 in the absence (lane 1) of the label or by incubation of the protein in the presence of Mg₈-8-N₃-3’-biotinyl-ATP in the dark (lane 2) did not result in labeling of NBD94, indicating the specific covalent binding of 8-N₃-3’-biotinyl-ATP by the protein.

A more detailed location of the label in NBD94 was determined by MALDI-TOF mass spectrometry. For that, the Mg²⁺-8-N₃-3’-biotinyl-ATP-labeled protein band was subjected to in-gel tryptic digestion. Fig. 3B shows a MALDI-TOF spectrometry map. The peptides were identified as bands derived from NBD94 (Table 2). In addition an increase of 1079 Da for the fragment 488EKLKHYNFDDFVK (500) (numbering includes the His₈ tag; peak position, 2761.85 m/z) was observed, suggesting that this peptide was labeled by 8-N₃-3’-biotinyl-ATP bound as a triethylammonium salt derivative.

Nucleotide Binding of NBD94 Determined by Fluorescence Correlation Spectroscopy—ATP and ADP binding of NBD94 and the strength of the nucleotide binding were further examined by FCS, which is a highly sensitive tool to determine binding/dissociation equilibria. The characteristic diffusion time, τₓ, for NBD94 was measured by binding of labeled nucleotides to the protein. The diffusion times of the NBD94 ATTO-647N complex were compared with the standard fluorophore Cyanine 5 and the ATTO-647N-conjugated nucleotides without NBD94. The mean count rate per ATTO fluorophore bound to NBD94 was 56.4 kHz for ATP ATTO-647N (50 nM) and 31.2 kHz for ADP ATTO-647N (50 nM), respectively. Fitting the autocorrelation functions resulted in characteristic times of diffusion τₓ = 62.4 μs for ATP ATTO-647N and τₓ = 62.7 μs for ADP ATTO-647N.

Fig. 4A shows the measured autocorrelation curves of the fluorescent ATP analogue, ATP ATTO-647N,
in the absence and presence of different concentrations of NBD94. The addition of protein resulted in a significant change in the mean diffusion time $t_D$. The mean diffusion time increased up to 14.9%. A binding constant of $647N$ was tested with the full-length protein NBD94 the mean diffusion time increased up to 14.2% with increasing concentrations of NBD94. This confirmed that nucleotides bound to NBD94 in the absence of Mg$^{2+}$ (ratio of 1:1). A binding constant of $31.6 \% $ for bound ATP ATTO-647N to NBD94 was calculated (Fig. 4C). When the ADP analogue ADP ATTO-647N was tested with the full-length protein NBD94 the mean diffusion time increased up to 14.9%. A binding constant of $26 \pm 3 \mu M$ for bound ADP ATTO-647N to NBD94 was determined (Fig. 4D).

NBD-Cl has been found to inhibit the $F_{1}F_{0}$-ATP synthase by reacting with a Tyr residue in the central nucleotide binding domain of the catalytic subunit $\beta$ (62), resulting in the loss of the lowest affinity binding site for nucleotides (63). By comparing the autocorrelation curves of the NBD94-ATP ATTO-647N-bound form with those in which the protein has been preincubated with increasing amounts of NBD-Cl, the diffusion time of ATP ATTO-647N was shown to decrease with increasing concentrations of NBD-Cl, indicating that nucleotide binding to NBD94 became inhibited.

Secondary Structural Alterations Due to Nucleotide Binding

Probed by Spectroscopy—To obtain additional information on possible secondary structure alterations in NBD94 after nucleotide binding the fluorescence emission of the protein with and without ligands was studied. Because NBD94 lacks any tryptophan residue the fluorescence spectrum was monitored using the intrinsic tryrosine fluorescence (Fig. 5, curve $\Delta$). Addition of MgADP (curve $\bigcirc$) decreased the quantum yield (up to 18%) with a concurrent shift of the maximum to a longer wavelength, which might be caused by the reorientation of a Tyr residue(s) inside the binding pocket to a more polar environment. The fluorescence intensity was further reduced in the presence of MgATP (curve $\square$). In contrast, NBD94 in the presence of MgGTP (curve $\times$) displayed a spectrum similar to that obtained in the absence of nucleotides. The data show that the tyrosine fluorescence spectrum of NBD94 is sensitive to ATP and ADP binding and offered the possibility to investigate the binding behavior of the protein to MgATP and MgADP. The results of both titration experiments are shown in Fig. 5B, which reveals a hyperbolic shape of the binding curve. A binding constant of $25 \pm 2$ and $31 \pm 2 \mu M$ was determined for MgATP and MgADP, respectively.

The secondary structure of NBD94 in the presence of nucleotides was determined from circular dichroism spectra (Fig. 2). Fig. 2 indicates that when MgATP is bound to NBD94 the protein showed a slightly lower helical content (62%) and a lower $\Theta_{222}/\Theta_{208}$ ratio (0.98). By comparison, the $\Theta_{222}/\Theta_{208}$ ratio of the MgADP form of NBD94 is 0.9, and the $\alpha$-helical amount is 69%. The identical values could be observed in repeated experiments. In summary the data imply significant secondary structural alterations due to nucleotide binding.

Purification and Spectroscopic Traits of the Truncated 65-kDa Form of NBD94—To determine what forms the minimal nucleotide-binding core of the NBD94 protein, the C terminus-truncated form of NBD94 (NBD94-(1–550)), including both the Walker A and B motifs, was constructed. The expressed protein was purified by metal chelate affinity chromatography followed by ion-exchange chromatography (DEAE; Fig. 6A). In the final step the protein was eluted from a size exclusion column (Superdex S-75 10/300 GL column) in a single peak. Analysis of the isolated protein by SDS-PAGE revealed the high purity of the 65-kDa protein NBD94-(1–550) (Fig. 6B). To explore the nucleotide binding traits of NBD94-(1–550) FCS experiments were performed. Fig. 4B illustrates the measured autocorrelation curves of MgATP ATTO-647N in the absence and presence of NBD94-(1–550). The addition of the truncated form resulted in a significant change of the mean diffusion time $t_D$, whereby the diffusion time of MgATP ATTO-647N increased up to 31.9%. A binding constant of $51 \pm 3 \mu M$ for MgATP ATTO-647N bound to this protein was calculated (Fig. 4C). By comparison, the diffusion time of MgADP ATTO-647N in the presence of the truncated protein yielded an increase of 31.6%. A binding constant of $53 \pm 3 \mu M$ for MgADP ATTO-647 bound to NBD94-(1–550) was established (Fig. 4D). The data showed that NBD94-(1–550) is still able to bind nucleotides albeit at a lower affinity compared with the entire NBD94 protein. In addition, when CD spectroscopy was used to elucidate the effect of MgADP or MgATP binding on the secondary structure of NBD94-(1–550) the spectra were found to be similar to that done in the absence of nucleotides (Fig. 7).

Impact of ATP/ADP on Erythrocyte Binding of Full-length Py235 Protein—To investigate whether ATP or ADP had a direct impact on the ability of Py235 to bind to erythrocytes, erythrocyte binding assays in the presence of either no nucleotide, ATP, or ADP were carried out (Fig. 8). Bound Py235 was detected using the well characterized monoclonal antibody

### Table 2: MALDI-TOF mass spectrometry analysis of peptides of NBD94

| Start residue | End residue | Measured mass | Sequence |
|---------------|-------------|---------------|----------|
| 1             | 18          | 2260.0941     | HHHHHHPPSDDKHEVYK |
| 24            | 38          | 1719.8911     | KVYVENNAYIDLAK |
| 52            | 64          | 1511.8315     | DKKYSTIKSELK |
| 60            | 82          | 2586.3344     | SALSAGYQDDALYNLESLIVK |
| 119           | 140         | 2557.8433     | LNLGLTSDKRENLLSLIIVMK |
| 161           | 180         | 2402.1470     | EKQSSNINDSYDSYKDNLK |
| 203           | 218         | 1987.9607     | DEDAKQNYEKSKEYK |
| 239           | 256         | 2125.1036     | DIDLKNYVNYFVNLNHHK |
| 275           | 293         | 2299.9849     | NIEQSDQNYDENDNISD |
| 302           | 314         | 1580.7082     | SIEEYQNNILT |
| 322           | 334         | 1568.8101     | ICNKTESIEKPR |
| 360           | 372         | 1546.7020     | SYTQFQDNLDR |
| 374           | 403         | 3579.4685     | TELEKIFTELGLSSEAYKNLNELYKDNLK |
| 398           | 410         | 1538.7849     | YFPNDKRENLOT |
| 404           | 431         | 3224.5342     | ENGLTPKNTLYQDFQENKATNDRIQ |
| 432           | 459         | 3187.6303     | SYVANNRSNIVTMHTSIY NIEDIK |
| 438           | 459         | 2574.3119     | NVSNDEYHIVSYTNYIDIEEK |
| 464           | 487         | 2815.5451     | NIELMKEILOEAEISYNENK |
| 488           | 500         | 2761.8536     | EKLLHYNFDPDV |
| 506           | 523         | 2149.1499     | YADEIKNRIKNDITLDQ |
| 524           | 537         | 1613.9584     | VDKRHALTEK |
| 559           | 571         | 1606.8322     | TYYNEQDKEIEE |
| 584           | 601         | 2227.1678     | YIDYNNKKKLIINIAEIE |
| 611           | 627         | 1999.0681     | NNNSYQGKLKLKPK |
| 635           | 647         | 1600.8073     | SBNKIRSKME |
| 699           | 702         | 1538.8006     | ESKLKLTAENYK |
| 695           | 710         | 1865.9603     | LTGKCNYKSNINDIK |
| 754           | 764         | 1444.7318     | EYTKKIEEYNK |
| 779           | 790         | 1379.6835     | TIERKANLCTK |

* Peptide labeled with 8- $N_3$-3-biotinyl-ATP.
25.77 (18) that recognizes the full length in the parasite supernatant (Fig. 8, lane 4). Binding of Py235 to erythrocytes could be inhibited by treatment of the erythrocytes with trypsin or chymotrypsin, whereas treatment with neuraminidase had no effect (data not shown), confirming the previously described binding properties of Py235 (13). The erythrocyte binding assay showed some binding of Py235 to erythrocytes in the absence of any additional nucleotide (Fig. 8A, lane 3). Binding of Py235 increased significantly in the presence of additional MgATP (Fig. 8A, lane 2), whereas there was a noticeable reduction of binding in the presence of additional MgADP as compared with no nucleotide added (Fig. 8A, lane 1). This was confirmed by
The initial interactions between the merozoite and the erythrocyte that lead to junction formation and invasion involve a cascade of receptor-ligand interactions. In *P. yoelii*, the Py235 rhoptry protein multigene family has been implicated in host cell selection and is strongly linked to parasite virulence. Clonal phenotypic variation of *py235* in *P. yoelii*, where each merozoite originating from a single schizont expresses a distinct *py235* member, has also implicated Py235 in playing a role in host cell adaptation and immune evasion (21, 22). Currently a single member of Py235 has been shown to directly bind to erythrocytes, and although at this stage it is believed that the other Py235 members also have red cell binding activity this still awaits confirmation (13, 64). The interaction of Py235 with the erythrocyte is mediated by a specific trypsin- and chymotrypsin-sensitive erythrocyte receptor (13); this is in line with the PfRH1 protein of *P. falciparum* that has been shown to bind to a specific erythrocyte receptor (14, 16). These findings indicate that part of the protein sequence of Py235 encompasses a receptor binding domain that mediates the specific interactions needed for erythrocyte recognition. A gap in our understanding is the molecular events that lead up to the binding of Py235 to its receptor and the subsequent events that lead to the downstream signaling that enables the merozoite to continue the invasion process. Considering the large size of Py235, it is likely that the full-length protein contains other functional domains besides a receptor binding region. Here analysis of the known Py235 protein sequences by bioinformatics approaches predicted the presence of a nucleotide binding domain with relatively high confidence. Importantly this domain was only predicted in all Py235 members and in other members of the RBL family. In addition, the NBD was not predicted for any other key malaria invasion protein we analyzed, indicating an important role of nucleotide binding in the function of RBL. Based on the initial prediction, we successfully expressed a 94-kDa domain of Py235 and showed that it indeed binds the nucleotides ATP and ADP with high specificity. Based on FCS and tyrosine fluorescence analysis, binding to ATP was stronger than that observed for ADP. Interestingly ATP binding was inhibited in the presence of NBD-Cl, a potent inhibitor of F-ATP synthases (65). In this case the inhibitor binds to a Tyr residue of one catalytic β subunit of F,F₅′-ATP synthases and prevents this so-called empty β subunit from being changed into a nucleotide-binding state (62). Because of the protein traits described above, we have termed our recombinant protein NBD94. There were significant changes observed in the intrinsic tyrosine fluorescence spectra upon binding of NBD94...
to either ATP or ADP, respectively, indicating that these two nucleotides induce slightly different structural changes in the adenine-binding site of the binding pocket upon nucleotide binding. Additional evidence for the changes in secondary structure comes from the CD analysis of NBD94 in the presence of either ATP or ADP. The changes in the spectra are consistent with a significant change in the helical contents of the secondary structure in response to binding either ATP or ADP. Using the truncated version NBD94-(1–550) that still retains the ATP/ADP binding activity although with slightly reduced affinity clearly showed that the observed structural changes due to nucleotide binding can be contributed by the C-terminal part of NBD94. Our findings are consistent with this C-terminal region acting like a hinge that changes conformation depending on whether ATP or ADP is bound. Such hinge structures have been described previously for the nucleotide-binding subunits β (66) and B (67) of the F- and A-ATP synthases, respectively, where the C-terminal domain of the nucleotide-binding subunit undergoes a relative rotation, effecting the coupling with the neighboring subunits γ and ε and subunit F, respectively, thereby resulting in rotational movements in the entire enzyme complex. From this, it is clear that NBD94 can assume three different structural states based on the presence of ATP, ADP, or no nucleotide consistent with the idea that NBD94 functions as an ATP/ADP sensor inside the Py235 complex. Importantly the observation that addition of ATP/ADP modulates the direct binding of Py235 to the erythrocyte strongly suggests that the conformational changes induced by these nucleotides regulate accessibility of the binding domain in the full-length protein.

The ability of Py235 to sense ATP adds another level of complexity to the already complex process of host cell recognition and invasion by the merozoite. Previous studies have indicated that intracellular ATP is important for merozoites to establish an infection inside the erythrocyte (32–35) possibly playing a role in the phosphorylation of the erythrocyte cytoskeleton (68). Within this context, it would be of benefit to the merozoite to detect whether an erythrocyte contained sufficient ATP to provide a suitable environment before committing to invasion. Secretion of ATP by erythrocytes occurs under normal physiological conditions but is significantly enhanced by mechanical deformation (29) as well as other stimuli like hypoxia and hypercapnia (69). This secretion of ATP from the erythrocyte would lead to the establishment of an ATP gradient that could be sensed by the merozoite. The very small difference in the binding affinities by NBD94 for ATP as compared with ADP indicates that small changes in the ratios of ATP/ADP such as would be expected to be observed at the sur-
face of the erythrocyte would trigger the conformational change observed in the study reported here. Such structural changes could serve as an activation step of Py235 that directly leads to Py235 binding to its erythrocyte receptor, thereby enabling the invasion process to proceed.

In summary, the data presented demonstrate for the first time that besides the already characterized signal peptide, erythrocyte binding properties, and transmembrane domain of the reticulocyte-binding protein Py235 this large multidomain protein also contains a nucleotide binding domain. The preference of MgATP over MgADP binding associated with specific structural alterations in the C-terminal domain of NBD94 implies its role as a mediator, which might facilitate the linkage of nucleotide signaling and Py235 binding to the blood cell. The finding of a nucleotide binding domain inside Py235 will be a stimulus for studying the detailed function(s) and interactions of the ensemble of domains within this important class of invasion proteins.

Acknowledgments—We thank Dr. Shahid Khan for introducing J. Ramalingam to the SMART program and for stimulating discussions and Dr. S. K. Sze for mass spectrometry analysis. We are grateful to Prof. H.-J. Schäfer (Johannes-Gutenberg University, Mainz, Germany) for the generous gift of the analogue 8-N3-3-biotinyl-ATP.

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