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***Plasmodium vivax* trophozoite-stage proteomes**

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Abstract

Plasmodium vivax is the causative infectious agent of 80–300 million annual cases of malaria. Many aspects of this parasite's biology remain unknown. To further elucidate the interaction of *P. vivax* with its *Saimiri boliviensis* host, we obtained detailed proteomes of infected red blood cells, representing the trophozoite-enriched stage of development. Data from two of three biological replicate proteomes, emphasized here, were analyzed using five search engines, which enhanced identifications and resulted in the most comprehensive *P. vivax* proteomes to date, with 1375 *P. vivax* and 3209 *S. boliviensis* identified proteins. Ribosome subunit proteins were noted for both *P. vivax* and *S. boliviensis*, consistent with *P. vivax*'s known reticulocyte host–cell specificity. A majority of the host and pathogen proteins identified belong to specific functional categories, and several parasite gene families, while 33% of the *P. vivax* proteins have no reported function. Hemoglobin was significantly oxidized in both proteomes, and additional protein oxidation and nitration was detected in one of the two proteomes. Detailed analyses of these post-translational modifications are presented. The proteins identified here significantly expand the known *P. vivax* proteome and complexity of available host protein functionality underlying the host–parasite interactive biology, and reveal unsuspected oxidative modifications that may impact protein function.

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Author contributions

Conceived and designed the experiments: DA, SL, SA, EM, JB, CK, MG. Performed the experiments: SL, SA, EM, CK, DA. Analyzed the data: DA, SL, SA, EM, JB, CK, MG. Wrote the paper: DA, MG.

Conflict of interest

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Biological significance—*Plasmodium vivax* malaria is a serious neglected disease, causing an estimated 80 to 300 million cases annually in 95 countries. Infection can result in significant morbidity and possible death. *P. vivax*, unlike the much better-studied *Plasmodium falciparum* species, cannot be grown in long-term culture, has a dormant form in the liver called the hypnozoite stage, has a reticulocyte host–cell preference in the blood, and creates caveolae vesicle complexes at the surface of the infected reticulocyte membranes. Studies of stage-specific *P. vivax* expressed proteomes have been limited in scope and focused mainly on pathogen proteins, thus limiting understanding of the biology of this pathogen and its host interactions. Here three *P. vivax* proteomes are reported from biological replicates based on purified trophozoite-infected reticulocytes from different *Saimiri boliviensis* infections (the main non-human primate experimental model for *P. vivax* biology and pathogenesis). An in-depth analysis of two of the proteomes using 2D LC/MS/MS and multiple search engines identified 1375 pathogen proteins and 3209 host proteins. Numerous functional categories of both host and pathogen proteins were identified, including several known *P. vivax* protein family members (e.g., PHIST, eTRAMP and VIR), and 33% of protein identifications were classified as hypothetical. Ribosome subunit proteins were noted for both *P. vivax* and *S. boliviensis*, consistent with this parasite species' known reticulocyte host–cell specificity. In two biological replicates analyzed for post-translational modifications, hemoglobin was extensively oxidized, and various other proteins were also oxidized or nitrated in one of the two replicates. The cause of such protein modification remains to be determined but could include oxidized heme and oxygen radicals released from the infected red blood cell's parasite-induced acidic digestive vacuoles. In any case, the data suggests the presence of distinct infection-specific conditions whereby both the pathogen and host infected red blood cell proteins may be subject to significant oxidative stress.

Keywords

Plasmodium vivax; Proteomics; Malaria; Trophozoite stage; Infected red blood cell; Protein oxidation/nitration

1. Introduction

Plasmodium vivax malaria is a serious neglected disease with transmission in 95 countries [1] and an estimated 80 to 300 million yearly cases, extreme morbidity and the possibility of death [2,3]. Infection typically results in repeated episodes of paroxysms, with high fever and chills, and symptoms that include violent headaches, vomiting, diarrhea, and muscle aches. Clinical parameters can also include an enlarged spleen, thrombocytopenia and severe anemia, and disease ramifications can be a particular concern for pregnant women [4]. As a significant public health threat, a detailed examination of this parasite's biology and biochemistry is warranted for the development of possible vaccines, diagnostics and therapeutics that can reduce disease burden [1–3,5,6]. It is important for such studies to proceed in parallel with the most lethal and better studied species, *Plasmodium falciparum*. These two most predominant malaria-causing species are phylogenetically distant [7], and species-specific interventions will be important for today's global efforts to control, eliminate and ultimately eradicate malaria [8].

For each species of *Plasmodium*, the expressed proteome during the parasite's life cycle stages in the mosquito vector and its primate host would be expected to have stage-specific differences. This is also the case as the parasite develops in the blood over an approximate 48-hour period from the ring stage of development to a growing trophozoite and through its schizogonic multiplication phase. The trophozoite stage of development is critical for the parasite to undergo morphological changes, grow in size, and remodel the host red blood cell (RBC) to suit its development and release of new infectious merozoite forms into circulation. During this stage, the parasite is also consuming host hemoglobin from within the RBC and processing the toxic hemozoin byproduct into inert pigmented hemozoin crystals known as hemozoin [9].

Importantly, unlike *P. falciparum*, which invades RBCs of all ages, *P. vivax* specifically invades the young RBCs known as reticulocytes [10,11]. *P. vivax*, and a few other species including the human malaria species *Plasmodium ovale* [12] and the closely related simian malaria model species *Plasmodium cynomolgi* then begin to synthesize caveolae vesicle complexes (CVCs) [13]. These are elaborate structures that develop around the entire infected host cell membrane with the caveolae cup-like portion externalized and the vesicular and tubular structures internal within the host cell cytoplasm [12]. The CVCs have been observed from *P. cynomolgi* in 3-dimensions using electron tomography and by immuno-electron tomography showing the PHIST/CVC-8195 protein localized to the outer portions of CVC tubules [14,15]. Many other parasite-encoded infected RBC (iRBC) membrane proteins have been identified by SDS-PAGE analysis of purified *P. vivax* infected RBC membranes from *Saimiri boliviensis* monkey infections, with several others associated with the CVCs and other iRBC membrane structures [15] but these have remained uncharacterized. Critically, *P. vivax* and *P. cynomolgi* lack the knob-like morphology characteristic of *P. falciparum* iRBC surface structures, and which are known for expression of adhesive variant proteins that are associated with virulence [3,5,15]. Thus, *P. vivax* and *P. cynomolgi* iRBC biology is very different from *P. falciparum* (and other species) in many important respects that remain largely unexplored. These biological differences include the expression in *P. vivax* (and *P. cynomolgi* [16]) of members of a multigene family called *vir*, which encodes several hundred small presumptive variant antigen proteins with multiple predicted localizations [17–19]. This is in contrast to the ~60 member *var* gene family in *P. falciparum* and the related ~108 member *SICAvar* family in *Plasmodium knowlesi*, with each confirmed to encode large variant antigens that become positioned at the surface of the infected RBCs and undergo switching events in the course of an immune response [20,21].

Basic studies of *P. vivax* iRBCs are especially challenging because, unlike *P. falciparum* iRBCs, *P. vivax* iRBCs cannot be cultured continuously in vitro, requiring their isolation from live hosts [22]. The first *P. vivax* parasite genome was reported in 2008 with 5459 genes, based on the Salvador I (Sal I) strain obtained from *S. boliviensis* monkey infections; ca. 3086 of these genes were annotated as hypothetical [23]. Preliminary proteomic studies of *P. vivax* blood-stage forms were reported in 2009 with the identification of 16 proteins from a single patient [24] and then 154 proteins in 2011 from a multi-patient pool of blood-stage isolates [25]. Roobsoong et al. [26] reported 314 proteins from cultured schizont stage-

enriched *P. vivax* iRBCs from pooled samples of multiple patients which also contained gametocytes. While this manuscript was in revision, Moreno et al. reported identification of 238 *P. vivax* (VCG-1 strain) trophozoite proteins, and 485 Aotus host proteins, in a sample containing 70% trophozoites [27]. [MRG1] Malaria patient serum protein changes [28,29] are dominated by acute phase response proteins or proteins linked to this non-specific inflammatory response, which can be induced by a variety of infections, tissue injury, trauma, cancer, stress, inflammation or immunological disorders [30]. However 44 *P. vivax* antigens were identified in the serum immunoproteome from 22 vivax malaria patients, with 5 being present in over 80% of patient sera [31]; these antigens, alone or in combination with selected acute-phase response proteins, could be a starting point for malaria diagnostics.

Stage-specific analyses of patient-isolated *P. vivax* iRBCs are complicated by the low abundance of parasites with typically low parasitemias (<1% infected host RBCs), blood draw limitations from sick patients, and the likelihood of an asynchronous composition of the life cycle stages, as well as potential for multiple broods and multiple strains. Pooling samples from patients will increase parasite yields but results in the increased likelihood (or inevitability) of multiple strains and assorted possible protein modifications in the analyzed samples. An alternative is the use of suitable non-human primate (NHP) experimental models, such as the Bolivian squirrel monkey *S. boliviensis* [5,32–34]. Using this model, specific *P. vivax* blood-stage infections can be optimized with adequate blood draws timed for the predominance of distinctive developmental stages; thus increasing the potential of identifying low and high abundance proteins, enabling the association of a greater number of proteins and their putative functions with individual stages of development, and beginning to associate specific protein modifications detected in distinct in vivo biological replicates with disease processes.

Here we present three *P. vivax* proteomes (Pv-Proteome 1, Pv-Proteome 2, and Pv-Proteome 3) from iRBCs enriched for trophozoites from *S. boliviensis* monkey [34] infections with the Sal I strain for which the *P. vivax* genome was first published [23]. We report in-depth analyses of two of these proteomes (Pv-Proteome 1 and Pv-Proteome 2) with the identification of 1375 *P. vivax* and 3209 host RBC proteins at a ~2% false discovery rate, based on multiple monkey infections, use of five different search engines for identifications, and assessment of unexpected post-translational modifications (PTMs) of both host and parasite proteins. As an alternative to indirect analysis of oxidative modifications by reaction of protein carbonyl groups with 2,4-dinitro-phenylhydrazine followed by western blot analyses [35], we directly examine the extent and heterogeneity of protein oxidation in more detail using tandem mass spectrometry. This study represents the most comprehensive identification of *P. vivax* trophozoite and host proteins to date in the context of *P. vivax* blood-stage infections, which is important for a systems biology examination of infected RBCs, changes in post-translational modifications, and pathogenesis.

2. Materials and methods

2.1. Pathogen isolation

P. vivax—Three independent *P. vivax* (Sal-1) blood-stage infections were initiated in *S. boliviensis* monkeys acquired from the MD Anderson breeding facility in Texas, which is supported by the National Institutes of Allergy and Infectious Diseases (NIAID). *S. boliviensis* infections were initiated by blood transfers from other infected *S. boliviensis* individuals, transferring 0.5–1.0 ml of blood with a parasitemia of 0.4–1%. The parasite density was estimated from analyses of thin blood smears. Infected blood for the three respective proteomes came from Saimiri monkeys named SB3609, SB3603 and SB3414. The infections were initiated with cryopreserved ring-stage iRBC stocks of *P. vivax* made available from the Centers for Disease Control and Prevention (CDC) and monitored based on specifications detailed in a protocol approved by Emory University's Institutional Animal Care and Use Committee (IACUC). These parasites had been passaged previously in splenectomized *S. boliviensis* to ensure adequate peak parasitemias (at least 1%) from this monkey adapted strain; thus, splenectomies were performed prior to infection to ensure comparable yields. When parasitemias were between 1.5–3% with mostly late trophozoite-stage parasites, blood was collected into sodium heparin tubes and processed through glass beads and a Plasmodipur filter using standardized procedures to remove platelets and white blood cells, respectively. The infected blood sample was then layered onto a 52% Percoll gradient to concentrate and purify samples that were enriched for trophozoites. The resulting iRBC parasite pellets (~1e9 parasites) for PvProteomes 1, 2 and 3 consisted of 91%, 71% and 89% trophozoite forms with the remaining parasites being young 2–4 nuclei schizonts and a low percentage of gametocytes. Specifically the trophozoite/2–4 nuclei schizont/gametocyte breakdowns for PvProteomes 1, 2 and 3 respectively were: 91%/8%/1%; 71%/29%/0%; and 89%/11%/0%. These iRBCs were frozen at –80 °C, and thawed at a later date for proteomic analyses.

Mycobacterium smegmatis—This mycobacterium was cultured in Middlebrook 7H10 medium according to [36], lysed by bead-beating, heat denatured in reagent grade 4 M urea and 10 mM dithiothreitol (both from Sigma-Aldrich, St. Louis MO) at 95 °C for 15 min in pH 8.0 0.2 M tris buffer, alkylated with 30 mM iodoacetamide (Sigma-Aldrich, St. Louis MO), serially proteolyzed with 1:30 by weight lysC endoprotease (Wako USA, Richmond VA) for 24 h, then by 1:30 by weight trypsin (Sigma-Aldrich, St Louis MO) for 24 h at 37 °C. Peptides were isolated and analyzed as below.

2.2. Proteome analysis

Pv-Proteome 1 and Pv-Proteome 2. *P. vivax* iRBC proteins and peptides were prepared for analysis using the FASP-I [Pv-Proteome 1] or FASP-II [Pv-Proteome 2] protocols [37], desalted using 100 µl OMIX C18 tips (Agilent, Palo Alto, CA) [Pv-Proteome 1] or 3 M Empore disk cartridges [Pv-Proteome 2], roughly quantitated using absorbance at 280 nm [37] on a Nanodrop spectrometer (Nanodrop, Wilmington, DE), and analyzed by 2D SCX (strong cation exchange)/C18 RP (reversed phase) LC/MS/MS on a Thermo Scientific (San Jose, CA) LTQ-XL ETD Orbitrap mass spectrometer with New Objective (Woburn, MA) PV-550 source [38]. Precursor ions were analyzed in the Orbitrap, and MS/MS spectra were

analyzed in the linear ion trap. For Pv-Proteome 1 this involved use of a 4 cm long IntegraFrit (New Objective Inc., Woburn, MA) 75 micron (μ) diameter capillary column packed with Polysulfoethyl Aspartamide 5 μ , 300 Angstrom pore diameter strong cation exchange resin in series with a 28 cm PicoFrit (New Objective Inc., Woburn, MA) 75 μ diameter capillary column self-packed with Jupiter 5 μ , 300 Angstrom pore C18 resin (Phenomenex, Torrance, CA).

Peptides were analyzed with top-10 CID fragmentation; precursor ions with 1+ or unassigned charges were rejected for fragmentation. Peptides were eluted from the capillary columns with an Agilent 1200 nano-HPLC at 300 nl/min. For Pv-Proteome 1, after loading ca. 1.1 μ g of peptides onto the SCX column, 14 individual salt steps eluted strong cation exchange column, consisting of 2 μ l each of 2.5, 5, 10, 20, 25, 30, 40, 50, 75, 100, 150, 200, 300 and 1500 mM pH $\sim$ 3 ammonium formate followed by a final elution with acetonitrile (15 fractions total). An internal lock mass for $[\text{Si}(\text{CH}_3)_3\text{O}]_6$ of 445.120024 was used for internal recalibration [39]. For Pv-Proteome 2, two separate 2D LC/MS/MS runs were concatenated for analysis, with 33 μ g and 22 μ g peptides loaded respectively onto a 28 cm $\times$ 75 μ i.d. strong cation exchange column in series with a) a 20 cm 5 μ particle C18 75 μ i.d. PicoFrit column, and b) a 10 cm 3 μ ReproSil-Pur 200 Angstrom pore C18-AQ resin (Dr. Maisch GmbH, Ammerbuch, Germany) 75 μ i.d. column. A total of 18 elutions of the SCX column used the above salt steps, with an addition of elution with water in the first step after loading, deletion of the 2.5 mM salt step, and addition of 15, 125, and 500 mM salt steps.

We used the PlasmoDB.org *P. vivax* release 7.1 database, downloaded March 15, 2011, and the NCBI *S. boliviensis* fasta protein database, downloaded April 3, 2014, for analysis. Data analysis utilized multiple search engines; an overview is included in Table 1 below. Andromeda (v. 1.2.0.14, embedded in Maxquant v. 1.2.0.18 software) [40] used default parameters of 20 ppm uncertainty for precursor ions in the initial search, 6 ppm uncertainty in the second search, and 0.5 Da uncertainty for MS/MS fragments. The peptide false discovery rate was 1%; identified proteins were included up to a PEP of 2%. Mass Matrix v. 2.4.0 [41] included proteins up to a false discovery rate of 1.73%. X!Tandem v. 10-12-01-1 [42] included proteins to an expectation value of 0.98, and had a peptide false discovery rate of 2.37%. Mascot [43] v. 2.3.02 with Mascot Distiller v. 2.4.2.0 included proteins up to a false discovery rate of 2.07% using Percolator scoring [44] of peptide spectrum matches (PSMs). SEQUEST [45] utilized Percolator peptide scoring embedded in Thermo Proteome Discoverer v. 1.3.0.339 software, with protein PEP maximally 2% (confidence of protein identification minimally 98%) calculated using custom Excel macros based on the Protein Prophet algorithm [46] without the mixture model. For protein identification, searches were generally conducted with a precursor ion tolerance of 13 ppm and product ion tolerance of 0.8 Da. Identifications from all search engines for Pv-Proteomes 1 and 2 are listed in Supplementary Tables 1A, B, C and D. Identification by a minimum of two different search engines [47] was utilized for consideration of the protein's function when assessing *P. vivax* or *S. boliviensis* biology. Different search engines used different algorithms for protein grouping; proteins are thus presented as individual proteins independent of groups, with information on individual search engine results presented in the Supplemental Tables 1A–

1D. Two pseudogenes, each tentatively identified by one search engine, were deleted from the list of identified proteins as both included numerous stop sites.

SEQUEST analysis of PTMs included only individual peptides with posterior error probabilities of 0.01 or less as scored by Percolator [44], a search engine rank of 1, and Preliminary Score (Sp, [39]) value of 200 or higher to avoid PSM with large unmatched peaks. For a less ambiguous examination of modifications, PSMs with a Delta Score ($Xcorr[2nd\ ranked\ peptide] - Xcorr[top\ ranked\ peptide] / Xcorr[top\ ranked\ peptide]$) of 1, e.g., PSMs with no second ranked peptide, were analyzed. Variable modifications in the initial database searches were carbamidomethyl cysteine and oxidized methionine; searches used strict trypsin specificity, and up to two missed trypsin cleavages were allowed. Mascot was searched in error tolerant mode to identify unsuspected peptide modifications by mass. Due to the combination of the Orbitrap's precursor ion high mass measurement accuracy with Percolator peptide scoring, these peptide modifications were then examined using SEQUEST searches with a variety of variable modifications.

To cover a large number of modified residues based on initial results, since SEQUEST in Proteome Discoverer 1.3.0.339 can only examine six variable modifications at one time; multiple parallel searches were run and then concatenated in Proteome Discoverer using Multireport. Variable modifications in these searches with monoisotopic mass additions in parentheses included: a) oxidation (15.9949 Da) and dioxidation (31.9898 Da) of C, M, F, H, W and Y, and trioxidation (48.9847 Da) of C and Y; b) nitration (44.9851 Da) of F, H, W and Y; c) nitrohydroxylation (60.97999 Da) of F, H, W and Y; d) oxidation of W to kynurenine (3.9949 Da); e) formation of 4-hydroxy-2-nonenal (HNE) adducts of C, H and K (156.1150 Da); f) formation of a tyrosine quinone (dopaquinone [42]) (13.9793 Da); g) oxidation of A, D, G, I, K, L, M, N, P, Q, R, T, V and dioxidation of I, K, L, M, P, R, V; and h) oxidation of tyrosine to topa quinone (29.9742 Da).

Estimates of relative site occupancy for an individual residue modification utilized spectral counting, where occupancy = [PSMs for peptide with that site modification] / [total PSM for any version of that peptide] from a single database search. All analyzed modified peptides had a Percolator PEP of 0.01 or lower, a preliminary score Sp of 200 or higher, search engine rank of 1, and Proteome Discoverer (v. 1.3.0.339) delta score of 1. To minimize false positive nitrotyrosine identifications, precursor mass measurement accuracy for these peptides was 5 ppm or better for these fully tryptic peptides [48].

Pv-Proteome 3. A preliminary proteome identifying 688 *P. vivax* iRBC proteins, obtained by SEQUEST analysis of LTQ-Orbitrap LC/MS/MS data at the Emory Microchemical Facility (functioning prior to 2009), is included in Supplemental Table 1E as Pv-Proteome 3. For this experiment, solubilized *P. vivax* iRBC samples were extracted with reducing SDS-PAGE sample buffer and resolved on 4–15% SDS-PAGE gradient gels, and the gels were then stained with colloidal Coomassie blue. Gel slices were excised, destained, dried, and processed as reported previously [49]. The gel pieces were digested with trypsin (Sigma; St. Louis MO) and the resulting peptides were extracted with trifluoroacetic acid (Sigma; St. Louis, MO). The samples were then desalted and concentrated using ZipTip pipette tips (Millipore; Billerica, MA). Cleaned peptides were analyzed by reverse-phase liquid

chromatography coupled with tandem mass spectrometry (LC–MS/MS) by an LTQ–Orbitrap mass spectrometer.

This initial analysis relied on at least two peptides to identify a protein, and used the *P. vivax* genome database as well as other Plasmodium species genome databases at NCBI for comparative assessments (Supplemental Table 1E). This dataset provides a broad overview of the *P. vivax* iRBC proteome with a predominance of trophozoite-stage proteins. These data were generated before the *S. boliviensis* genome sequence was available; thus *S. boliviensis* identifications are not included. This analysis also did not include analysis of PTMs, as shown here for Pv-Proteomes 1 and 2, or posterior error probabilities (PEPs) supporting the peptide/protein identifications. Overlaps of identified proteins from Pv-Proteome 3 with Pv-Proteomes 1 and 2 (251 proteins in common) are included in Supplemental Fig. 1 and Supplemental Table 1F. We have not compared the identifications from Pv-Proteome 3 to those of Pv-Proteomes 1 and 2 in more detail due to significant differences in the data analysis.

3. Results

3.1. 2D LC/MS/MS identification of *P. vivax* and *S. boliviensis* proteins

Fig. 1 shows Giemsa-stained trophozoite-stage *P. vivax*-infected iRBCs from *S. boliviensis* infections after purification using a Percoll gradient. Using such purified *P. vivax* iRBC preparations, with a predominance of trophozoites as described in detail in the Materials and methods section, we aimed to identify proteomes from multiple biological replicates. To increase the number of identifications, peptides from two trophozoite-enriched samples were analyzed using five different search engines (SEQUEST, Mascot, Andromeda, Mass Matrix and X!Tandem) (Fig 2A and B). In Pv-Proteome 1, the first of two proteomes analyzed by this approach, 459 *P. vivax* (Supplemental Table 1A) and 1533 *S. boliviensis* (Supplemental Table 1B) proteins were identified by at least one search engine with a ~2% false discovery rate (Fig. 2A and B). Sequest, Mascot and X!Tandem contributed the most unique identifications. In Pv-Proteome 2, for which a larger amount of peptide was analyzed (55 ug vs 1.1 ug for Pv-Proteome 1), 1262 *P. vivax* (Supplemental Table 1C) and 2078 *S. boliviensis* proteins (Supplemental Table 1D) were identified by at least one search engine. In Pv-Proteome 1, Sequest identified the most proteins, and the most proteins unique to a single engine, while X!Tandem identified the most proteins and unique proteins for Pv-Proteome 2. Fig. 2C illustrates Venn diagrams for protein identifications from both of these proteomes; 344 *P. vivax* proteins and 400 *S. boliviensis* proteins are common to both proteomes. Data from a preliminary *P. vivax* proteome (Pv-Proteome 3) is included in Supplemental Table 1E, and is compared with Proteomes 1 and 2 in Supplemental Table 1F and Supplemental Fig. 1; 251 proteins identified are common to all three proteomes.

Fig. 3A and B illustrates the functional categories of 1109 *S. boliviensis* and 609 *P. vivax* proteins identified by 2D LC/MS/MS, in combined Pv-Proteomes 1 and 2, by at least two different search engines. Many proteins can have multiple functions; the function for each is assigned to what seems to be the most prominent functional category. The functional categorizations are based on current annotations in PlasmoDB, Uniprot, KEGG, Entrez, or publications in PubMed. The detailed protein identification lists for each functional group

are presented in Supplemental Table 2. The source of the functional annotation for some proteins, for which this is not obvious from the protein description, is listed in a separate “annotation” column in Supplemental Table 2. Functional annotation as “transcription” includes RNA polymerase complex proteins, while “RNA processing” annotation includes RNA polyadenylation, capping and splicing, and RNA transport to the cytoplasm. Annotation as “translation” includes ribosome assembly proteins, ribosomal proteins, and proteins involved in elongation on tRNA and termination. Fig. 3C illustrates the functional categories (using the same categories as above) for the *P. falciparum* trophozoite-stage proteome obtained by Prieto et al. [50]. This figure also presents the fraction of expressed proteins in each category for *P. vivax*, compared to *P. falciparum*, as a ratio (e.g., proteins involved in transport are shown as the same fraction (ratio of 1.00) of identified proteins in *P. vivax* vs. *P. falciparum*). PlasmoDB, Entrez [51], HMMER 3.0 [52], BlastP [53], InterProScan [54] and Pubmed were utilized to examine the potential annotation of proteins without any listed function. The largest differences in relative expression levels include a higher percent of *P. vivax* surface, cytoskeletal and translation-related proteins, and relatively fewer DNA replication/repair and hypothetical proteins.

Four categories account for ca. 67% of *P. vivax* identifications, including 203 proteins annotated as hypothetical proteins or conserved hypothetical proteins of unknown function, 92 proteins associated with translation including 58 ribosomal proteins, 59 metabolism-related proteins and 59 cell surface proteins (Fig. 3A). *S. boliviensis* combined Pv-Proteomes 1 and 2 include the additional functional categories of proteins involved with the host immune response, serum or extracellular proteins, hemoglobin-related proteins, structural proteins, actin-related cytoskeletal or signaling proteins, and proteins related to apoptosis (Fig. 3B). *S. boliviensis* redox-related proteins identified in combined Pv-Proteomes 1 and 2 include 5 thioredoxin-related proteins, 5 peroxiredoxin-related proteins, 2 glutaredoxins, 5 glutathione-related metabolic enzymes, 2 superoxide dismutases and catalase. Identified *P. vivax* redox-related proteins include a 2 peroxiredoxins, 2 glutaredoxins, thioredoxin, superoxide dismutase, glutathione reductase, and merozoite capping protein 1, and a putative thiol peroxidase protecting cells against reactive oxygen species toxicity [54].

The most abundant *P. vivax* and *S. boliviensis* proteins as calculated by Mascot [43] using the exponentially multiplied protein abundance index (emPAI, [55]) are listed in Supplemental Table 3. For *P. vivax* these include a conserved hypothetical protein with a histidine-rich membrane protein domain, five enzymes (triosephosphate isomerase, pyruvate kinase, phosphoglycerate kinase, lactate dehydrogenase, and aldolase) involved in or coupled to glycolysis, heat shock proteins (HSP) such as HSP70, HSP86 and GRP78, the redox proteins thioredoxin and 2-cys peroxiredoxin, and PHIST protein PVX_093680 (PHIST/CVC-8195 [14] which was detected as a high abundance protein in all three proteomes and with the highest relative abundance emPAI score of 8.0 in Pv-Proteome 2, consistent with the originally observed abundance of this protein in SDS Page gels [15]). This PHIST protein is a constituent of the iRBC's caveola vesicle complexes (CVCs) and is predicted to be critical for *P. vivax* survival [14]. Other abundant proteins include PVX_096070, which is an eTRAMP [56] that is present in all three proteomes, and several

hypothetical proteins also in all three proteomes. Three proteins annotated as VIR were also detected in all three proteomes with an emPAI relative abundance of 0.09 in the case of PVX_096980 and PVX_096985, and lower for the VIR8 related protein PVX_096970; these gene identifications were re-classified recently as cluster 12 non-VIR proteins [17]. Overall, there were only a few VIR or VIR-like proteins detected in addition to those referenced above; these include PVX_090305 and PVX_022185. Highly abundant *S. boliviensis* proteins identified include hemoglobin alpha, beta, and gamma subunits, actins and actin binding proteins, histones or histone-domain containing proteins, redox enzymes including thioredoxin, peroxiredoxins, superoxide dismutase and glutathione peroxidase, several glycolytic enzymes, and several HSPs. High levels of several ribosomal subunits were also identified from both *P. vivax* and *S. boliviensis*.

3.2. Protein post-translational modifications

For initial examination of peptide PTMs, we utilized a second-pass error-tolerant search after Mascot's initial search, which indicates modifications by mass shifts from unmodified peptides. Results indicated extensive oxidation in Pv-Proteome 1. Due to the availability of Percolator scoring for individual modified peptides, modifications were then examined with SEQUEST in multiple searches specifying a variety of oxidative modifications. Only peptides with a Percolator posterior error probability of 0.01 or below, a search engine rank of 1, and a preliminary score (Sp, [45]) above 200 were accepted for analysis. Table 2 presents examples of different residue oxidative modifications observed in Pv-Proteome 1 (columns 1–2) and Pv-Proteome 2 (middle two columns). In Pv-Proteome 1, ca. 79% of methionines were oxidized to methionine sulfone, and 16% oxidized to sulfoxides. Of 16 tryptophans only one was unmodified; 11 were singly or doubly oxidized and three were nitrated. Ca. 53% of tyrosines were nitrated, a little over 6% were singly or doubly oxidized, and 1.8% were nitrohydroxylated. Nitrohydroxylation has been reported for tryptophan [57] but not to our knowledge for tyrosine or phenylalanine. Ca. 15% of the 39 cysteines were oxidized to cysteine sulfonic acid; most were carboxamidomethylated as part of sample preparation. Of the 252 histidines present, 8% were oxidized or doubly oxidized and 0.4% were nitrated.

In contrast, peptide residues in Pv-Proteome 2, as analyzed above, were oxidized or nitrated to a lesser degree (Table 2, middle two columns). Only 1.6% of methionines were present as sulfones, and only 2.9% of tyrosines, 9.6% of tryptophans, 2.0% of phenylalanines and 3% of histidines were modified; 0.7% of cysteines were oxidized to cysteine sulfonic acid.

To obtain a comparison of PTMs, an available soluble *M. smegmatis* proteome [36] was also evaluated. This proteome was selected since: 1) peptides were prepared and data was acquired under conditions similar to those of Pv-proteomes 1 and 2; 2) the cultured *M. smegmatis* were not exposed to a host immune response; and 3) *P. vivax* itself cannot be cultured. Oxidative modifications of *M. smegmatis* tryptic peptides, analyzed as above, are summarized in the far right two columns of Table 2. As with Pv-Proteome 2, a) the fraction of methionines oxidized to sulfones is low (6.5%) compared to Pv-Proteome 1 although ~77% are oxidized to methionine sulfoxide; b) nitrotyrosine residues comprise less than 1% of tyrosines vs. ~52% in Pv-Proteome 1; c) no cysteine sulfonic acids are observed; and d)

oxidized histidines are less than 1% of the total histidines. Oxidized phenylalanine is highest in Pv-Proteome 1 (6% of total residues), lowest in the *M. smegmatis* proteome (~1%) and intermediate in Pv-Proteome 2 (2%). As with the Pv-Proteome 1, over 20% of *M. smegmatis* tryptophans are mono-oxidized, roughly 10-fold higher than in the Pv-Proteome 2. However the Pv-Proteome 1 has almost half of 16 tryptophans doubly oxidized, compared to ~1–2% for the other two proteomes.

Table 3 shows details of oxidized peptides for three of the more extensively modified proteins, *S. boliviensis* hemoglobin and actin, and the *P. vivax* PHIST/CVC-8195 protein, which is a major component of the parasite's CVCs that are predicted to be critical for *P. vivax* survival [14]. Both hemoglobin alpha and beta chains appear modified at a number of residues, although the fractional modification at individual sites, calculated by identical database searches for each proteome and using spectral counting and dynamic modifications, varies over a ~100-fold dynamic range. These modifications may be underestimated, as the sequence coverage for each protein is incomplete. In Pv-Proteome 1, hemoglobin residues such as Y10 of peptide EFTPQVQAAYQK, W7 of peptide AAVTALWGK, Y2 of peptide TYFPHFDLSHGSAQVK, 6 residues of the PHIST/CVC-8195 protein, and six residues of two actins appear to be hot spots for modification. In Pv-Proteome 2 many of the same modifications are present at the same sites, but at lower frequencies. Some residues, such as Y10 of peptide EFTPQVQAAYQK, Y8 of peptide VGSHAGDYGAEALER, and F1 of peptide FLASVSTVLTSK, can have several different modifications. As shown in Table 3, *S. boliviensis* actin is extensively oxidized in Pv-Proteome 1, with some oxidation sites (e.g., Y240) reported to be involved, when oxidized, in forming disorganized filaments [58].

Table 4 lists some additional *P. vivax* proteins containing oxidized or nitrated residues, as identified by SEQUEST. One large category of modified proteins in Pv-Proteome 1 includes HSPs, chaperones and redox-related proteins. In addition to well-annotated HSPs, these include the conserved hypothetical protein PVX_117795 with an HSP90-binding domain and an HSP23-like domain [59]; the p23-hBind-1 like domain may bind Rac1, activating NFkB and JNK signaling. A second hypothetical protein, PVX_090900 has strong sequence homology to a thioredoxin from *Toxoplasma gondii*, as well as homology to the HSP DnaJ [53]. A second large category includes translation-related proteins, such as ribosomal subunits and elongation factors. Peptides from these proteins are most commonly modified by methionine oxidation to the sulfone, tyrosine nitration, and oxidation/hydroxylation of other residues. Many of these proteins were also identified in Pv-Proteome 2, where the most common modifications were methionine oxidation and tyrosine nitration. Nitrated and oxidized/hydroxylated *S. boliviensis* proteins are listed in Supplemental Table 4. As with *P. vivax*, major categories include HSPs, proteins associated with protein folding or redox-related proteins; energy and metabolism proteins; and translation-related proteins. Modified cytoskeletal proteins include actins and actin-related proteins such as transgelin-2, profilin and actin-related protein 2. Other cytoskeletal proteins such as tubulins, myosins, vinculin and vimentin are also modified. Smaller functional categories are also listed.

Fig. 4 shows representative MS/MS spectra for a number of oxidative modifications of the *S. boliviensis* hemoglobin alpha subunit peptide VGSHAGDYGAEALER. The top three

peptide spectra were obtained from Pv-Proteome 1, and bottom 3 spectra from Pv-Proteome 2. Spectra of Y8-nitro and Y8-nitrohydroxy peptides are in the next two spectra below the unmodified peptide spectrum (top). These three peptides have the same +3 charge. Nitrohydroxylation has been reported for tryptophan [57] but not for tyrosine or phenylalanine; here we observe that tyrosine and (in other peptides) phenylalanine can also undergo this modification. The lower three MS/MS spectra of Fig. 4, all from +2 precursor ions, identify peptides with mono-, di- and tri-oxidized tyrosine. Based on spectral counts, the relative abundances of the modified Pv-Proteome 1 Y8-nitro and Y8-nitrohydroxy peptides compared to the unmodified peptide are 0.68 and 0.12 respectively (Table 3); for the less-oxidized Pv-Proteome 2, the relative abundances of the Y8-oxidized, -dioxidized and -trioxidized peptides are ~ 0.01 , 0.001 and 6.8×10^{-5} , respectively. Supplemental Fig. 2A shows MS/MS spectra of the hemoglobin beta subunit peptide VVAGVANALAHK, comparing the peptide with unmodified, oxidized and dioxidized histidine. Supplemental Fig. 2B shows MS/MS spectra of the hemoglobin beta subunit peptide AAVTALWGK, comparing peptides with tryptophan oxidized, dioxidized, nitrated, and nitrohydroxylated. These results and those in Table 3 illustrate that a number of different oxidative modifications of aromatic residues can occur in what can be a strongly oxidizing environment for some host and pathogen proteins.

4. Discussion

P. vivax enriched trophozoite-iRBC proteomes, with a total of 1607 parasite proteins identified from three proteomes, have been presented from multiple biological replicates, with the intent of investigating parasite biology and interactions involving the host reticulocyte proteins that may be pertinent to malaria pathogenesis and revealing possible targets of intervention. This is the most in-depth analysis of any ex vivo iRBC *P. vivax* proteome, which includes a variety of PTMs that may represent the result of biochemical reactions associated with the host-parasite interactions and the pathophysiological dynamics of infection. We have used five search engines, and biological replicates, to attain as much information as possible that may provide insights not only on the presence of proteins, but the engagement of the immune response and pathophysiology. Such information is considered relevant and will become increasingly applicable in systems biological models of malaria [60], including those being developed by the Malaria Host-Pathogen Interaction Center (MaHPIC, [61]).

The well-established [5,32,62] *S. boliviensis* *vivax* malaria model allows isolation of life cycle stage-enriched iRBC, with blood draws after infection timed to allow for the isolation and enrichment of specific stages such as trophozoites. This overcomes difficulties obtaining stage-specific proteomes from human patients harboring asynchronous life cycle stages and/or multiple *P. vivax* strains when patient samples are pooled. However it is possible that the identified host proteome (and even some expressed *P. vivax* proteins) may differ between *S. boliviensis* and human host iRBC. While this manuscript was in revision, Moreno-Prez et al. reported the identification of *P. vivax* (VCG-1 strain) blood-stage protein proteomes, which included trophozoite proteins (238), and Aotus host proteins (485), in a sample containing 70% trophozoites [27]. Together, these reports provide

confirmation of the value of capitalizing on the use of the available small New World monkey models [22] for *P. vivax* research.

The reported lack of correlation between mRNA transcripts and expressed protein levels [63–65], including in *P. falciparum* [66,67] makes it clear that examination of *P. vivax* stage-specific biology and functional genetics requires the evaluation of both transcriptome [68] and proteome data. This is true with regard to understanding the intra-erythrocytic development cycle (IDC) of the parasites growing and multiplying within RBCs over the course of their 24–72 h (depending on the species), but also in terms of pathogenesis and immune evasion strategies that are the result of antigenic variation mechanisms [20]. We have shown that the presence of SICAvir transcripts alone is not necessarily an indicator of the subsequent expression of the encoded SICA proteins in *P. knowlesi* and that the spleen plays a role in transcriptional or post-transcriptional regulatory processes [21]. Whether similar tactics govern certain gene and protein expression mechanisms in *P. vivax* remains unknown. For example, our detection of very few VIR proteins from among the several hundred vir genes present in the genome could be indicative of a process that regulates their restricted expression and the spleen may likewise be required to up-regulate and maintain the expression of these (and possibly other) transcripts and proteins in *P. vivax*, as has been suggested for *P. falciparum* expression of surface iRBC antigens [69]; it is thus possible that the proteome may be altered in splenectomized animals. Alternatively, members of this family may be expressed at levels too low for us to detect in these proteomes, or they may be expressed at other stages in the life cycle. Since the spleen is part of the mononuclear phagocyte system and is involved in removal of iRBC, hemoglobin and heme metabolism, it is possible that splenectomy may alter the immune response seen in non-splenectomized patients, and may thus affect the observed peptides and their metabolic modifications.

For this analysis, we have combined high-resolution fourier transform MS, an internal mass standard to give high precursor ion mass accuracy [39] and improved modification analysis, online 2D peptide separation to maximize identifications, machine learning analysis [70]-based Percolator [44] scoring to give improved peptide and peptide modification identifications, and use of multiple combined search engines [47] to increase the depth of the identified proteome. To limit exclusion of low abundance proteins [55], we included proteins identified by a single unique peptide if the proteins met overall criteria summarized in Table 1.

The Pv-Proteome 1 analysis was based on ~1.1 µg of tryptic peptides while the analysis of Pv-Proteome 2 was based on ~55 µg in two 2D LC/MS/MS runs; this large difference in peptide amounts available may account for the larger number of proteins identified in Pv-Proteome 2. For both *P. vivax* and *S. boliviensis*, the identification of many unique proteins by different search engines, and overlaps between the two proteome biological replicates of ~23% and ~9%, respectively, suggest that additional proteins are expressed for each organism. The exclusion of numerous proteins below the ~98% confidence level, many of which are likely expressed, also supports the premise that the actual expressed proteomes are in fact significantly larger than identified here. The combined *P. vivax* trophozoite-enriched proteome from these two biological replicates of 1375 proteins at a false discovery rate of ~2% is comparable to the largest *P. falciparum* trophozoite proteome of 1253

proteins, published in 2009 with a false discovery rate of 5% [50], and to the first *P. falciparum* trophozoite proteome, reported in 2002, of 952 proteins [71]. This represents the expression of ~25% of 5419 *P. vivax* reported gene clusters [72]. This is comparable to the ~1050 transcripts detected in the transition from trophozoite to early schizont stage [73], and the percentage of *P. vivax* trophozoite-stage expressed genes as mRNA transcripts reported from patient isolates [68] but there is not a direct mRNA-expressed protein correlation in this work or in other studies [64–67] that can be due to dynamics of translation and protein degradation [67]. Moreover, identified proteins from our study may include some proteins from other stages since Pv-Proteomes 1 and 2 represent between ~71% and 91% trophozoites.

For a more global view of trophozoite-stage biology, we examined proteins expressed by both *P. vivax* and the *S. boliviensis* host, and have also compared *P. vivax* and published *P. falciparum* protein expression. The combined *S. boliviensis* iRBC Pv-Proteomes 1 and 2 total of 3209 proteins is substantially larger than the 842 protein human RBC membrane proteome, which itself had overlaps ranging only from 29–53% with other published RBC membrane proteomes [74]. In Pv-Proteomes 1 and 2 we observed 26 of the 34 *P. falciparum* [32]-ring/early trophozoite stage-predicted 40S ribosomal subunits, and ~35 of 40 predicted 60S subunits, suggesting fairly consistent expression of ribosomal subunits in both species. Ten of the 40S subunits and seven 60S subunits are observed for both *P. vivax* and *S. boliviensis*. The proteome reported here is comparable in size to the current human RBC proteome of 2289 unique proteins [75]. Proteomes of the mature RBC, which is anuclear, contain only a few ribosomal proteins [76,77], which are thought to be left over from the reticulocyte development stage [76]. Our identification of numerous ribosomal proteins is thus consistent with the *P. vivax* infected host RBCs being reticulocytes and not mature RBCs. It is interesting to note that combined *P. vivax* proteomes 1, 2 and 3 identified here share a core of 53 proteins in common with the *P. vivax* schizont proteome [26] and with a reported *P. vivax* human patient clinical proteome [24] (Suppl. Table 1G and Suppl. Fig. 3); this core is enriched in proteins involved in metabolism, heat shock, stress response and protein folding, and translation, functions that would be expected to be common to multiple stages.

We included an available proteome from in vitro cultured *M. smegmatis* for comparison of the extent of oxidized residues, as no oxidation from a host immune response would come into play in this instance. *P. vivax* iRBCs cannot be cultured in vitro, and thus a direct comparison with ex vivo iRBC samples is not feasible; however such comparisons of NHP ex vivo derived and in vitro culture-derived iRBCs can be carried out with *P. knowlesi* or *P. falciparum* and these would be of interest in future investigations. Likewise, follow-up studies based on other life stages of *P. vivax* are of high importance and will provide a strong comparison of the many proteins expressed at different points in the parasite's development and relevant for different aspects of its survival.

Pv-Proteomes 1 and 2 have significantly different levels of oxidized and nitrated protein. Oxidized cysteine and aromatic residue frequencies in Pv-Proteome 2 appear similar overall to those in a cultured control *M. smegmatis* proteome (Table 2), with a higher fraction of methionine present as methionine sulfoxide in the mycobacterial proteome. Nitrotyrosine

and nitrophenylalanine levels are low in both proteomes. Methionine can be oxidized to methionine sulfoxide in a variety of conditions; e.g., with aging RBCs [78], under oxidative stress [79], in plasma proteins from patients with inflammatory disorders [80], or in proteins from activated neutrophils [81]. This oxidation is reversible by the enzyme methionine sulfoxide reductase coupled to thioredoxin, thioredoxin reductase, NADPH and the cellular redox system [82]. Froelich and Reid [83] observed that 4% of methionines were oxidized to sulfoxides in LC/MS/MS analysis of in-solution tryptic digests, while 3% of tryptophans were also oxidized. The presence of trace divalent or trivalent metal ions can catalyze methionine oxidation in solution [82]. Given the potential variability of methionine sulfoxide levels with preparation details and storage, observed differences could be due to one or more of these factors.

Pv-Proteome 1 in contrast contains substantially higher levels of oxidized and nitrated peptides than Pv-Proteome 2; many of these derivatives have been observed in other systems [84]. The Orbitrap mass spectrometer was operated with an electrospray voltage of 2.1 kV and silica capillary columns, which should avoid electrospray-induced corona discharge-dependent protein/peptide oxidation seen at higher (3.5 kV) spray voltages when using stainless steel capillary columns, but not observed at 2.0 kV [85]. Under these spray conditions we normally do not observe significant methionine oxidation or methionine sulfone in peptides. In Pv-Proteome 1, methionine in peptides with a PEP of 0.01 or less is present mostly as methionine sulfone. Oxidation of methionine to methionine sulfone in vitro requires strong oxidizing agents such as chloramine T or performic acid, neither of which were present here; thus we have concluded that the methionine sulfone levels observed may reflect physiologically pertinent endogenous events. The existence of methionine sulfones in proteins is unusual but has been observed in the oxidatively damaged DJ-1 protein in autosomal recessive Parkinson's disease, and in Alzheimer's disease [86].

In Pv-Proteome 1, 79% of methionines are oxidized to the sulfone and 15% of cysteines are present as cysteine sulfonic acid (Table 2). In the context of iRBCs these may be irreversible modifications and could affect protein function; other oxidation products such as cysteine, cysteine glutathione adducts, or cysteine sulfenic acid can be reduced by dithiothreitol and may thus be poorly represented here [87].

Over half of the tyrosines are present as nitrotyrosine. Tyrosine can be oxidized to nitrotyrosine by peroxynitrite or $\cdot\text{NO}_2$ [88,89]. The peroxynitrite is generated from nitric oxide and superoxide anion from the NADPH oxidase system [90]; the nitric oxide can originate from activated macrophages or endothelial cells. Tyrosine can be hydroxylated [91]; hydroxytyrosine can be present as DOPA (3,4-dihydroxyphenylalanine), which has been identified in mitochondrial proteins and may be a more common marker of oxidative stress than nitrotyrosine [48,91]. Tyrosine can also be derivatized para- to the phenolic hydroxyl by reaction with superoxide, to give (after reduction) a different hydroxytyrosine structure than DOPA [92]; these cannot be distinguished by our measurements. Phenylalanine can be oxidized by hydroxyl radicals, to o-, m- or p-hydroxyphenylalanine (tyrosine), oxidized by peroxynitrite to nitrophenylalanine [88], or doubly oxidized to produce DOPA; we have observed mono- and dioxidized phenylalanine derivatives as well as nitrophenylalanine. Oxo-histidine can be produced by attack of singlet oxygen [93] or by

hydroxyl radicals [80]. By added mass, we have observed this species as well as dioxidized histidine and nitrohistidine.

Some unusual modifications observed here (Table 2), all with relative stoichiometries of 0.06 or less, include nitrohydroxylation, which has been reported for tryptophan [57] but not for tyrosine or phenylalanine to our knowledge; both are observed here. In HIV patients with encephalitis, nitrohydroxylated tryptophan observed in immunoglobulin variable regions has recently been linked to the immune response [94]. Hydroxylation but not dihydroxylation of histidine and tyrosine has been reported [80]; here we observe dioxidation/dihydroxylation of a number of residues, particularly in Pv-Proteome 1, including tyrosine, tryptophan, phenylalanine, and histidine. The selectivity of protein residue modification varies inversely with the reactivity of the reactive oxygen species, with nonselective agents such as hydroxyl radicals attacking most accessible amino acids [80], while singlet oxygen attacks tryptophan, tyrosine, histidine, methionine and cysteine [93]. Highly reactive free radicals such as hydroxyl radicals have a short (~2 ns) lifetime and will derivatize proteins only within ca. 20Å of their cellular source [82]. Thus proteins modified by these radicals on normally unreactive residues such as glycine, alanine, or leucine, such as hemoglobin and actin, may reside close to the source of the radicals. Less reactive species such as nitric oxide can modify residues (e.g., cysteine) over much longer distances.

There are several potential sources of reactive oxygen species that can cause in vivo damage to iRBC proteins. First, neutrophils and macrophages can produce superoxide anions, hydrogen peroxide and hypochlorous acid in oxidative bursts (reviewed in [84]) that are part of the immune response to pathogens. The host immune response to malaria can include production of nitric oxide and oxygen radicals [95]. Both monocytes and neutrophils from *P. vivax* patients can be highly activated, with neutrophils showing enhanced superoxide production [96]. Hydrogen peroxide can be converted to hydroxyl radicals by metal-catalyzed oxidation systems, e.g. NADPH and NADH oxidases, xanthine oxidase, and cytochrome p450 reductase/oxidase (reviewed in [84]). Hydroxyl radicals can oxidize many amino acid side chains (vide supra) [80]. However the trophozoites observed in Fig. 1 do not seem to be visibly damaged, thus a destructive immune response seems less likely. Second, in iRBCs, proteolytic hemoglobin degradation in acidic digestive vacuoles produces free Fe^{3+} -heme, which can promote production of toxic oxygen radicals [97,98]. Electron transfer can occur to oxidized heme groups from protein side chains, resulting in formation of tyrosine, tryptophan, histidine, and cysteine radicals that can lead to their subsequent modification [80].

A highly oxidizing environment documented in *P. falciparum*-infected iRBC [95] which is thought to be due to food vacuole Fe^{3+} -heme mediated oxidation, has been reported to result in oxidative carbonylation of chaperones, proteases, and proteins involved in energy metabolism such as glycolytic enzymes [35]. In glucose-6-phosphate dehydrogenase-deficient erythrocytes carrying the African A- allele, thought to enhance protection against malaria by oxidative damage resulting in enhanced phagocytosis [100] of iRBC [95], *P. falciparum*-infected iRBC exhibited oxidative damage of traffic/assembly of cytoskeleton and surface proteins, stress response proteins, and oxidative stress defense proteins [101]. In blood group O individuals, thought to be protected against severe malaria compared to

individuals with other blood groups, *P. falciparum*-infected iRBCs exhibited a steady increase in 4-hydroxy-2-nonenal oxidative protein carbonylation during trophozoite maturation, with carbonylation of lipid raft and cytoskeletal proteins [102]. A differential pattern of carbonylation of cytoskeletal proteins was observed compared to A, B and AB groups, which may correlate with protection against severe malaria. This oxidative stress has been linked to *P. falciparum* malaria pathology [95] in addition to enhanced phagocytosis. Major categories of oxidized *P. falciparum* iRBC proteins include chaperones, proteins important for trafficking and assembly of cytoskeletal proteins, metabolism and glycolysis, protein synthesis/translation, redox proteins, and membrane/cell surface proteins [35,101,102]. We have identified oxidation of both host and pathogen proteins in each of these categories (Table 4; Suppl. Table 4). This is consistent with our identifications potentially reflecting iRBC oxidative biology common to both *P. vivax* and *P. falciparum*.

Although we have detected significant oxidation in only one of the two iRBC proteomes included here, we speculate that when it occurs (and if even transient in the context of dynamic biological systems), there may be significant functional consequences, as reported for the oxidizing environment of *P. falciparum* iRBC [60,95,98–102]. Modulation of signaling may involve reversible oxidations, such as methionine sulfoxide formation (vide supra), cysteine oxidation to disulfide bonds or formation of glutathione [60] or cysteine sulfenic acid derivatives; methionines in proteins may function as endogenous antioxidants [103]. Note that some proteins expressed in Pv-Proteomes 1 and 2 are important for defense against oxidative modifications (Supplemental Table 2), including *P. vivax* peroxiredoxin, thioredoxin, superoxide dismutase and merozoite capping protein 1, and *S. boliviensis* glutaredoxins, peroxiredoxins, thioredoxins and related proteins, catalase, and superoxide dismutases (26 in total). Methionine sulfoxide formation is reversible but can also damage protein function [82]. Tyrosine nitration may contribute to redox regulation, by interfering with tyrosine phosphorylation, and potentially as a reversible modification [87]. Irreversible oxidation to methionine sulfone or cysteine sulfonic acid, or other modifications in Table 2, may result in long-lasting damage and modification of function of the oxidized protein or its signaling pathway.

In *P. falciparum* trophozoite-stage iRBCs, actin is remodeled to allow controlled trafficking of cargo vesicles important for functioning of Maurer's clefts and knobs [104], and can be oxidized [35,101,102]. Blood group O-derived hemoglobin variants somehow interfere with this remodeling and the establishment of a parasite-directed actin cytoskeleton in the infected cells [105]. In a rat model of oxidative stress, after use of x-irradiation to induce reactive oxygen species, actin was extensively oxidized, with partial oxidation of methionines including met-82 sulfone, oxidation of two of four tryptophans, and oxidation of several cysteines [106]. The oxidized actin exhibited decreased polymerization and a lower level of actin-activated myosin ATPase activity. We observe oxidation of numerous actin-associated proteins, and of several actins, including mono-, di- and trioxidized tyrosine, mono- and dioxidized methionine and tryptophan, dioxidized phenylalanine, nitrotyrosine, dopaquinone, and nitrohydroxyl-tyrosine (Table 3, Supplemental Table 4). Nitration of tyrosines 91, 198, and 240 was associated with disorganized filamentous mouse actin [58]; here we observe both oxidation and nitration of Y240 in the peptide

SYELPDGQVITIGNER. Oxidation of methionines 44, 47 and 82, observed *in vivo* in a rat model of oxidative stress (above), was also observed here. The PHIST protein PHIST/CVC-8195 [14] is oxidized at 5 different methionines and nitrated at 2 different tyrosines; the functional consequences of these modifications remain to be determined. Thus *P. vivax*-iRBC actin in Pv-Proteome 1, with similar as well as different modifications than reported, may also have modification-perturbed function, although details await experimental examination.

5. Conclusions

In this paper, we have examined *P. vivax* iRBC proteomes, enriched for the trophozoite-stage of development, using 2D LC/MS/MS and five search engines for analysis. Specifically, 1607 parasites and 3209 host proteins were identified. Identification of host proteins is not often emphasized, but here substantially larger numbers are identified than in previous *P. vivax* proteomes, consistent with infection of host reticulocytes and the biological complexity of this stage. One proteome reflects substantial oxidation and nitration of hemoglobin, actin and other host proteins. Oxidized/nitrated *P. vivax* proteins include PHIST/CVC-8195 and two other PHIST proteins, actin, a number of heat-shock and redox related proteins, metabolic enzymes and translation-related proteins. Oxidation/nitration in the second proteome is limited more to hemoglobin chains. Although host neutrophil-, macrophage-, or endothelial cell-mediated oxidation/nitration of pathogen proteins can be part of the host immune response, it is possible that oxidized heme groups, for example generated from hemoglobin proteolytic digestion in acidic digestive vacuoles, also contribute to the observed modifications. The highly oxidizing environment we observed in one proteome is consistent with reports of a similar environment in *P. falciparum* iRBC. Based on identified sites of oxidation or nitration it is possible that these modifications, if occurring *in vivo*, may have significant effects on the function of modified proteins such as actin.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

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Abbreviations

2D LC/MS/MS	two dimensional high performance liquid chromatography/tandem mass spectrometry
RBC	red blood cell

iRBC	infected red blood cell
CVC	caveolae vesicle complex
NHP	nonhuman primate
SCX	strong cation exchange
RP	reversed phase
CID	collision-induced dissociation
PSM	peptide-spectral match
emPAI	exponentially multiplied protein abundance index
NO	nitric oxide
ppm	parts per million
Xcorr	SEQUEST cross-correlation coefficient
Sp	SEQUEST preliminary score
z	charge
PEP	posterior error probability
HSP	heat shock protein
DOPA	3,4-dihydroxyphenylalanine

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Fig. 1.
An example of Giemsa-stained *P. vivax* trophozoite-enriched iRBCs after Percoll gradient purification. Ca. 1×10^9 iRBC-parasites were isolated as discussed in the Materials and methods section, containing between 71% and 91% trophozoites [MRG8] in PvProteomes 1–3.

Fig. 2. Analysis of *Plasmodium vivax* and *Saimiri boliviensis* trophozoite-stage proteomes from two biological replicates, using five database search engines. For Pv-Proteomes 1 and 2, a maximum false discovery rate of ca. 2%, maximum PEP of ca. 2%, or minimum protein expectation value of 98% were used for listing of the results from each search engine. Pv-Proteome 2 was generated with a larger quantity of peptides, which may explain the larger identified proteome for each organism. Analysis of the *P. vivax* proteome, which consists of 459 identified proteins (Pv-Proteome 1) or 1262 proteins (Pv-Proteome 2). For many search engines, the number of identified proteins is roughly doubled in the second proteome. B. Analysis of the *S. boliviensis* proteome, which consists of 1533 proteins (PvProteome 1) or 2078 proteins (Pv-Proteome 2). The five engines contributed relatively evenly to identifications in the first proteome; X!Tandem contributed the most identifications to Pv-Proteome 2. C. Comparison of protein identifications in Pv-Proteomes 1 and 2 for *P. vivax*

(top) and *S. boliviensis* (bottom). A total of 1375 *P. vivax* proteins, and a total of 3209 *S. boliviensis* proteins, were identified in the combined two proteomes.

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Fig. 3. Functional categorization of proteins expressed in the *P. vivax* and *S. boliviensis* trophozoite-stage, identified for combined Pv-Proteomes 1 and 2 by at least two different search engines. A. Functional annotation of *S. boliviensis* proteins used annotations in the *S. boliviensis* NCBI fasta database, Uniprot, KEGG, Entrez or publications in PubMed. The largest categories include proteins related to metabolism, transcription, translation, and 144 cytoskeletal proteins including 80 involved in the actin-related cytoskeleton. Other categories include 98 signaling proteins, and 76 proteins involved in intracellular trafficking.

Many proteins may have more than one function, thus this chart provides a rough overview of the functional categories of identified proteins. B. Functional annotation of *P. vivax* proteins using the Plasmo DB database, as well as the above databases when needed. The largest category (33% of identified proteins) is comprised of proteins with no annotated function, including both hypothetical and conserved hypothetical proteins. Other major categories included translation (92 proteins), metabolism, surface, proteolysis-related, and heat shock/protein folding related proteins. C. Functional annotation (as above) for published *P. falciparum* trophozoite-stage proteins [50], for which 46% of proteins identified at the 95% confidence level had no assigned function. *Plasmodium vivax* had 33% of proteins (~98% confidence level) with no assigned function, giving a vivax/falciparum ratio of 0.72 for proteins in this category. Similar vivax/falciparum ratios are listed for each category. The largest relative differences between *P. vivax* and *P. falciparum* included translation, surface protein annotation, and cytoskeletal proteins.

Fig. 4.

Tandem mass spectrometry (MS/MS) spectral assignments of representative oxidized/hydroxylated or nitrated peptides identified by SEQUEST, utilizing Percolator scoring. Labeled peaks are matched if they are within 0.8 Da of a predicted y or b ion; to simplify the labeling, matches for neutral loss peaks are not indicated. Matched y-ions are indicated in blue, matched b-ions are indicated in red, unmatched peaks are gray. Spectra illustrate five different oxidative tyrosine modifications for the *S. boliviensis* hemoglobin alpha subunit peptide VGSHAGDYGAEALER. The spectrum of the unmodified peptide is shown at the top of the figure. For each peptide the precursor mass errors from the theoretical mass, Sequest-derived cross-correlation coefficient Xcorr, and Percolator-derived peptide posterior error probability PEP are: unmodified (0.14 ppm, 4.23, 4.8e-5), Y8-nitro (0.31 ppm, 5.03, 9.7e-10), Y8-nitrohydroxy (0.24 ppm, 4.38, 6.1e-9), Y8-oxidized (-4.5 ppm, 3.00, 5.3e-4), Y8-dioxidized (-1.75 ppm, 2.79, 3.7e-3), Y8-trioxidized (0.72 ppm, 2.90, 2.7e-3). The spectrum y-axis for the last two peptides is expanded to show details of ion assignments. The precursor masses, and mass shifts of y- and b-ions including the modified tyrosine (when present), are consistent with modification on tyrosine.

Table 1

Overview of search engine protein identification.

Search engine	Protein identification	Limit
SEQUEST	Protein PEP	Maximum 2%
Mascot	Protein false discovery rate	Maximum 2.07%
Andromeda	Protein PEP	Maximum 2%
Mass matrix	Protein false discovery rate	Maximum 1.73%
X!Tandem	Protein expectation value	Minimum 0.98%

Oxidized and nitrated residues in *P. vivax* + *S. boliviensis* proteomes compared to an *M. smegmatis* proteome. ^a

Table 2

Proteome 1	Proteome 2		<i>M. smegmatis</i>	
2213 peptides	Fraction	981 pep	Fraction	5668 pep
653 met		189 met		866 met
0 reduced	0.000	109	0.577	146
101 sulfoxide	0.155	77	0.407	664
516 sulfone	0.790	3	0.016	56
335 tyr		373 tyr		1625 tyr
153 unmod	0.457	362	0.971	1585
12 oxidized	0.036	6	0.016	30
10 dioxidized	0.030	3	0.008	1
6 NO ₂ OH	0.018	0	0.000	0
177 NO ₂	0.528	2	0.005	9
16 trp		42 trp		633 trp
1 unmod	0.063	38	0.905	492
4 oxidized	0.250	1	0.024	133
7 dioxidation	0.438	1	0.024	8
3 NO ₂	0.188	1	0.024	0
1 NO ₂ OH	0.063	1	0.024	0
39 cys		149 cys		272 cys
30 CAM- <i>b</i>	0.769	147	0.987	272
1 oxidized	0.026	0	0.000	0
1 dioxidized	0.026	0	0.000	0
6 trioxidized	0.154	1	0.007	0
1 unmodified	0.026	1	0.007	0
779 phe		485 phe		2289 phe
748 unmod	0.960	475	0.979	2266
9 oxidized	0.012	7	0.014	19
20 dioxidized	0.039	3	0.006	2
6 NO ₂ OH	0.008	0	0.000	1

Proteome 1		Proteome 2		<i>M. smegmatis</i>	
2213 peptides	Fraction	981 pep	Fraction	5668 pep	Fraction
1 NO ₂	0.003	0	0.000	1	0.0004
249 his		366 his		1921 his	
230 unmod	0.924	355	0.970	1911	0.995
10 oxidized	0.040	4	0.011	10	0.005
11 dioxidized	0.044	7	0.019	0	0.00
1 NO ₂	0.004	0	0.000	0	0.00

^a Modifications are from SEQUEST searches allowing the variable oxidative modifications listed; all peptides have a Percolator posterior error probability of 0.01 or less; the same peptide with different modifications is counted as a separate peptide; nitrated peptides with mass deviations from the theoretical mass above 5 ppm have been removed [42]

^b CAM, carbamidomethyl.

Table 3

Oxidation of hemoglobin and other proteins.

Proteome 1			Proteome 2			Representative		
Hemoglobin beta chain	Modification	Frequency ^d	Peptide PEP <i>b</i>	Hemoglobin beta chain	Modification	Frequency ^d	Peptide PEP <i>b</i>	Representative
VVAGVANALAHK ₁₄₄	None	0.84	1.00E-07	VVAGVANALAHK ₁₄₄	None	0.999	1.30E-03	
	H11(O)	0.084	1.60E-04		H11(O)	0.001	8.40E-03	
	H11(O ₂)	0.076	6.50E-07					
VVAGVANALAHKYH ₁₄₆	Y13(O)	1.00	3.00E-05	VVAGVANALAHKYH ₁₄₆	None	0.20	7.60E-03	
EFTPVQAAAYQK ₁₃₂	None	0.22	1.70E-06	EFTPVQAAAYQK ₁₃₂	Y13(O)	0.80	1.70E-03	
	Y10(NO ₂)	0.60	1.00E-06		None	0.995	3.50E-04	
	Y10(NO ₂ OH)	0.092	3.60E-06		Y10(O)	0.005	7.10E-03	
	Y10(O)	0.048	3.50E-06	FFESFGIDLSTPDVVMNNPK ₆₀	None	0.338	3.40E-06	
	Y10(O ₂)	0.044	3.50E-04		M15(O)	0.619	1.90E-04	
GTFAQLSELHCDK ₉₅	H10(O ₂), C11(CAM) ^d	0.52	2.10E-06		M15(O), F2(O)	0.040	5.60E-03	
	C11 (O ₃)	0.39	2.10E-05		M15(O), F1(O ₂)	0.003	7.80E-04	
	H10(O), C11(CAM)	0.044	4.50E-05		C11(CAM)	0.992	6.80E-03	
	F3(O ₂), H10(O ₂), C11(CAM)	0.015	3.00E-03		C11 (O ₃)	0.002	1.10E-03	
	F3(NO ₂ OH), H10(O ₂), C11(CAM)	0.0074	1.90E-03		H10(O ₂), C11(CAM)	0.003	9.80E-04	
AAVTALWGK ₁₈	W7(O)	0.51	3.20E-05		F3(O), C11(CAM)	0.003	6.70E-03	
	W7(O ₂)	0.42	1.10E-04	AAVTALWGK ₁₈	None	0.920	6.40E-04	
	W7(NO ₂ OH)	0.058	4.40E-04		W7(O)	0.045	7.80E-03	
	W7(NO ₂)	0.044	2.70E-04		W7(O ₂)	0.009	2.10E-03	
VLGAFSDGLTHLDNLK ₈₃	None	0.84	3.40E-13	VLGAFSDGLTHLDNLK ₈₃	W7(NO ₂)	0.026	8.20E-03	
	H11(O)	0.16	5.60E-05		None	0.963	5.30E-04	
					F5(O)	0.028	1.30E-03	
Hemoglobin alpha chain					H11(O ₂)	0.010	1.30E-03	

Proteome 1				Proteome 2				Representative	
Hemoglobin beta chain	Modification	Frequency ^a	Peptide PEP ^b	Hemoglobin beta chain	Modification	Frequency ^a	Peptide PEP ^b	Frequency ^a	Peptide PEP ^b
VGS HAGDYGAEALER ₃₂	None	0.41	5.30E-07	VGS HAGDYGAEALER ₃₂	None	0.989	2.60E-03	0.989	2.60E-03
	Y8(NO ₂)	0.28	2.30E-12		Y8(NO ₂)	0.005	1.50E-05		
	Y8(O)	0.14	6.20E-07		Y8(O)	0.005	3.10E-03		
	Y8(O ₂)	0.073	1.70E-04		Y8(O ₂)	0.001	3.00E-04		
	Y8(NO ₂ OH)	0.051	1.20E-09						
	H4(O ₂), Y8(O)	0.024	4.10E-05		LLSHCLLVTLAAHHPAEFTPAVHASLDK ₁₂₈				
	H4(O ₂), Y8(NO ₂)	0.016	3.00E-10		C5(CAM), H4(O)	0.50	1.30E-03		
	H4(O ₂) Y8(O ₂)	0.0027	2.90E-03		C5(CAM), H4(O ₂)	0.50	1.80E-03		
	M1(O ₂)	0.84	1.20E-04		None	0.777	8.50E-03		
	M1(O ₂), F5(O)	0.10	8.60E-05		M1(O)	0.221	6.90E-04		
MFLSFPTTK ₄₁	M1(O)	0.038	3.10E-04	MFLSFPTTK ₄₁	None	0.002	9.90E-03	0.002	9.90E-03
	M1(O ₂), F5(O ₂)	0.011	4.70E-03						
	M1(O ₂), F2(NO ₂ OH)	0.0054	9.90E-03		TYFPHFDLSHGSAQVK ₅₇	0.935	1.00E-03		
					Y2(NO ₂)	0.033	1.00E-04		
	Y2(O)	0.82	4.40E-09		F3(O)	0.022	7.80E-03		
	Y2(O ₂), F3(O ₂)	0.18	3.60E-03		H10(O ₂)	0.009	6.20E-03		
					Y2(O ₂), F3(O ₂)	0.0011	9.90E-03		
	None	0.97	2.60E-08		None	0.992	2.50E-03		
	F1(O)	0.027	1.80E-05		F1(O)	0.008	1.40E-03		
	F1(NO ₂ OH)	0.0038	1.20E-05		VADALGTAVAHVDDMPNALSALSDLHAHK ₉₁				
FLASVSTVLTSK ₁₄₀				FLASVSTVLTSK ₁₄₀	None	0.081	6.80E-05	0.081	6.80E-05
					M15(O)	0.89	5.20E-05		
					M15(O ₂)	0.020	1.90E-05		
					M15(O), H28(O ₂)	0.00027	2.30E-04		
					Actins				
					SYELPDGQVITIGNER ₂₅₄	1.00	2.80E-04		
VADALGTAVAHVDDMPNALSALSDLHAHK ₉₁				VADALGTAVAHVDDMPNALSALSDLHAHK ₉₁				0.94	1.00E-06
	M15(O ₂)	0.94	1.00E-06						
	H26(O)	0.06	4.60E-05						
Actins ^c				Actins ^c				0.19	7.20E-08
SYELPDGQVITIGNER ₂₅₄				SYELPDGQVITIGNER ₂₅₄				0.58	3.60E-10

Proteome 1			Proteome 2			Representative	
Hemoglobin beta chain	Modification	Frequency ^a	Peptide PEP ^b	Hemoglobin beta chain	Modification	Frequency ^a	Peptide PEP ^b
DLYANTVLSGGTTMYPGIADR ₃₁₂	Y2(O ₂)	0.032	2.30E-03	DLYANTVLSGGTTMYPGIADR ₃₁₂			
	M14(O ₂)	0.21	1.70E-13		None	0.33	2.80E-03
	M14(O ₂), Y15(NO ₂)	0.21	1.10E-06		M14(O)	0.67	6.50E-05
	Y3(NO ₂), M14(O ₂) Y15(NO ₂)	0.21	1.50E-03				
	M14(O)	0.16	1.00E-13		None	1.00	7.20E-03
DLYANNVLSGGTTMYPGIADR ₃₁₄	Y3(O), M14(O ₂) Y15(NO ₂)	0.11	1.10E-10	VAPEEHPVLLTEAPLNPK	None	1.00	7.90E-04
	M14(O), Y15(NO ₂)	0.11	2.80E-09				
	Y3(O ₂), M14(O ₂)	0.50	1.60E-07		None	1.00	5.80E-03
	Y3(O ₂), M14(O)	0.25	7.70E-04				
	Y3(O ₂), M14(O ₂), Y15(NO ₂)	0.25	2.30E-04				
EITALPSTMK ₃₂₆	M10(O)	0.54	6.50E-05	YPIEHGHTNWDDMEK ₈₄			
	M10(O ₂)	0.46	5.10E-05		None	1.00	5.30E-03
	W11(O ₂), M14(O)	0.50	5.20E-04				
	W11(O), M14(O)	0.25	6.90E-03				
	W11(O ₂), M14(O ₂)	0.25	9.40E-03				
DSYVGDEAQS ₆₁	None	0.75	8.70E-05	PHIST/CVC-81 ₉₅			
	Y3(NO ₂)	0.25	8.30E-07		None	1.00	6.40E-04
	M5(O ₂), M8(O ₂)	1.00	3.60E-03				
HQQVMVGMGQK ₅₀				VIDENMPYPNGPFR ₄₅₂			
					None	1.00	1.10E-03
					None	0.80	2.10E-03
					M5(O)	0.20	8.70E-03
					None	1.00	3.80E-04
PHIST/CVC-81 ₉₅ (PVX_093680)				LEMEDDAFGSR ₆₂₇			
AELQEQMTEEELNSK ₆₇₁	M7(O ₂)	0.75	2.20E-09	LEMEDDAFGSR ₆₂₇			
	M7(O)	0.25	1.50E-06				
	M6(O ₂), Y8(NO ₂)	1.00	5.30E-09				
	M3(O ₂), Y8(NO ₂)	0.67	3.50E-04				
	M3(O)	0.33	2.60E-04				
LEMEDDAFGSR ₆₂₇	M3(O ₂)	0.50	2.60E-03				
	M3(O)	0.50	1.10E-04				

Proteome 1				Proteome 2				Representative	
Hemoglobin beta chain	Modification	Frequency ^a	Peptide PEP ^b	Hemoglobin beta chain	Modification	Frequency ^a	Peptide PEP ^b	Frequency ^a	Peptide PEP ^b
SEQIAAMNYEEQFHQGPR ₄₈₈	M7(O ₂)	1.00	8.40E-03	SEQIAAMNYEEQFHQGPR ₄₈₈	None	0.40	1.50E-06		
7 other peptides	None	1.00	to 9.9E-03	17 other peptides	M7(O)	0.60	8.30E-04		
					None	1	to 9.9E-03		

^aRatio of (site + modified peptide-spectral matches)/total peptide-spectral matches in the same search for the same site, for peptides with posterior error probability PEP < 0.01.

^bPEP as calculated by Percolator.

^cPeptides are from *S. boliviensis* cytoplasmic 1 or gamma enteric actin.

^dCAM, carboxamidomethyl.

Table 4

Examples of *P. vivax* trophozoite-stage oxidized or nitrated proteins.^a

Protein	Accession #	Proteome 1 sequence coverage %	Proteome 1 modifications	Proteome 2 sequence coverage %	Proteome 2 modifications
<i>Heat shock/protein folding/redox related</i>					
hsp86	PVX_087950	25.7	met(O, O ₂), tyr(NO ₂), phe(O ₂ , NO ₂), ala(O)	33.6	met(O)
78 kDa glucose-regulated protein	PVX_099315	21.2	tyr(NO ₂), trp(O ₂)	25.6	met(O)
hsp70 interacting protein	PVX_079865	5	met(O, O ₂), tyr(NO ₂)	6.9	
hsp70	PVX_089425	21.9	met(O, O ₂), tyr(NO ₂), phe(O ₂), ala(O)	23.9	met(O), thr(O)
hsp60	PVX_095000	14.1	tyr(NO ₂)	9	
T-complex protein 1 gamma subunit	PVX_124100	8.1	tyr(NO ₂)	4.6	
Conserved hypothetical protein ^b	PVX_117795	12.8	met(O ₂)	7.9	
Conserved hypothetical protein thioredoxin, DNA J analog	PVX_090900	6.5	met(O ₂)	4	
Thioredoxin	PVX_117605	37.5	met(O ₂)		
<i>Metabolism</i>					
Pyruvate kinase	PVX_114445	15.3	met(O, O ₂)	31.5	met(O, O ₂)
Lactate dehydrogenase	PVX_116630	30	met(O ₂), tyr(NO ₂)	32	met(O)
Aldolase	PVX_118255	23.3	tyr(NO ₂)	16.5	
Enolase	PVX_095015	17.7	met(O ₂), tyr(NO ₂)	39	met(O)
Triosephosphate isomerase	PVX_118495	14.1	phe(O ₂)	30	
Phosphoglycerate mutase	PVX_091640	8.8	met(O ₂)	30	met(O)
Hexokinase	PVX_114315	7.9	met(O ₂)	22.5	
<i>Translation</i>					
Elongation factor 1 alpha	PVX_114830	26.4	met(O ₂), cys(O ₂), lys(O, O ₂), leu(O ₂)	37	met(O)
Elongation factor 1 gamma	PVX_082845	5.1	phe(O, O ₂)	12.7	
Elongation factor 2	PVX_117925	8.3	met(O, O ₂), tyr(NO ₂)	15.6	met(O)
Nascent polypeptide associated complex alpha chain	PVX_114205	18.5	met(O ₂)	28.3	met(O)
hnRNP U	PVX_101610	7.4	met(O, O ₂), tyr(NO ₂)		
RNA binding protein	PVX_094535	10	met(O, O ₂), tyr(NO ₂)	10.8	met(O)

Protein	Accession #	Proteome 1 sequence coverage %	Proteome 1 modifications	Proteome 2 sequence coverage %	Proteome 2 modifications
40S ribosomal protein S3	PVX_117170	9.5	met(O ₂)	12.6	
60S ribosomal protein L10a	PVX_118430	18.4	met(O ₂), tyr(NO ₂)	18	met(O)
60S ribosomal protein P0	PVX_092120	17.5	met(O), tyr(NO ₂), NO ₂ -OH	14.6	met(O)
<i>Surface</i>					
Merozoite surface protein 7	PVX_082645	10.3	met(O ₂)		
Phist protein Pf-fam-b	PVX_093680	26.6	met(O ₂), tyr(NO ₂),	44.5	met(O)
Phist protein Pf-fam-b	PVX_112110	7.8	met(O ₂), tyr(NO ₂)	15	
Phist protein Pf-fam-b	PVX_088830	4.7	met(O ₂)		
<i>Other</i>					
Actin	PVX_101200	22.1	met(O ₂), tyr(NO ₂), quinone)	18.4	
Chloroquine resistance protein Cg4	PVX_087970	9	met(O ₂)	11.3	
Pv-fam-d protein	PVX_121910	10.7	met(O ₂)	9.3	
RAD protein (Pv-fam-e)	PVX_101610	7.5	met(O ₂)		
Conserved hypothetical protein ^c	PVX_115450	23.5	met(O ₂)	34.1	met(O)
Conserved hypothetical protein ^c	PVX_083560	16	met(O ₂), tyr(NO ₂)	23.7	met(O)
Conserved hypothetical protein ^c	PVX_083270	5.4	met(O ₂), tyr(NO ₂)	13.7	
Hypothetical protein	PVX_083555	29.6	met(O), tyr(NO ₂)	35.9	
Hypothetical protein	PVX_081830	12.1	tyr(NO ₂)	13.3	tyr(O)

^a All modifications are on peptides with a 1% or lower posterior error probability; cys carbamidomethylation is not shown.

^b HSP23 co-chaperone; HSP90 co-chaperone.

^c Protein has no published function.