Use of Biotin Derivatives to Probe Conformational Changes in Proteins

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Sulfosuccinimidyl-6-(biotinamido) hexanoate and derivatives thereof covalently bind to the e-amino group of lysine residues. Our observation that access of the biotin derivative to specific lysine residues depends on conformational properties of the entire polypeptide chain prompted us to investigate whether differential biotinylation patterns of a protein can be used as indicators for conformational changes. Bovine serum albumin is a soluble protein with characteristic unfolding kinetics upon exposure to high temperature. First, we show that biotinylation patterns of proteins are highly reproducible. Second, we demonstrate by mass spectrometry and tandem mass spectrometry that unfolding of the protein correlates with the accessibility of the biotin derivative to specific lysine residues. We have applied this experimental strategy to the analysis of a cell-surface protein, viz. the human band 3 anion exchanger of erythrocytes infected with the malaria parasite Plasmodium falciparum. We found that Lys826 in a highly flexible loop can be biotinylated in non-infected (but not infected) erythrocytes, confirming earlier observations (Winograd, E., and Sherman, I. W. (2004) Mol. Biochem. Parasitol. 138, 83–87) based on epitope-specific monoclonal antibodies suggesting that this region undergoes a conformational change upon infection.

The biotinylation of proteins is a commonly used tool for the affinity purification of proteins (1). It takes advantage of the highly specific interaction between biotin and avidin or its bacterial homolog, streptavidin. Protein biotinylation utilizes biotin derivatives containing an active ester group that reacts with primary amines of proteins, particularly with the N terminus and the e-amino group of lysine residues. A novel amide bond is thereby formed, and the biotin moiety is covalently attached to the respective amino group, often separated by a spacer of defined size. The biotin derivative sulfosuccinimidyl-6-(biotinamido) hexanoate (sulfo-NHS-LC-biotin) is a hydrophilic reagent that is excluded from most cells and is therefore used for the biotinylation and subsequent affinity purification of cell-surface proteins.

Recently, we used sulfo-NHS-LC-biotin to label proteins exposed on the surface of human red blood cells infected with the malaria parasite Plasmodium falciparum (iRBC) (2). In contrast to the non-infected erythrocyte (RBC), the plasma membrane of the infected erythrocyte was permeable for this biotin derivative (2). Uptake of the compound occurs through the so-called novel permeability pathways, which are parasite-induced and have a broad specificity with a preference for anions (3–6). Biotinylation of internal proteins can be minimized if the reaction occurs in the presence of novel permeability pathway inhibitors such as furosemide. The proteins that constitute the novel permeability pathway are still unknown. The band 3 anion exchanger AE1 is the most abundant anion transporter in RBC (7). The reactivity of an epitope-specific monoclonal antibody with band 3, which differs between infected and non-infected erythrocytes, has been interpreted as a conformational change in the protein in at least one of the extracellular domains (8). Although band 3 itself does not appear to be the actual channel protein mediating the novel permeability pathway (5), its conformational change as a consequence of the infection may nevertheless play an important role in the survival of the parasite within its host cell. Therefore, we sought an experimental strategy to more precisely define the position within the band 3 molecule where this conformational change could possibly occur.

In a recent proteome analysis, we used in situ biotinylation of permeabilized iRBC to specifically label soluble parasite proteins contained in the parasitophorous vacuole (9). Peptide mass fingerprinting of 27 different biotinylated proteins revealed a high reproducibility of the biotinylation patterns of individual proteins in different experiments. These observations prompted us to investigate whether changes in protein conformation are reflected by changes in biotinylation patterns and whether this mapping can be exploited as an experimental strategy to detect conformational changes in cell-surface proteins. Although amino acid residue modifications by specific tags and cross-linkers in combination with mass spectrometry
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have been used as tools for probing the tertiary structure of proteins (10–12), the use of biotin derivatives has additional advantages: (i) labeled proteins can be readily visualized before analysis; (ii) labeled peptides can be affinity-enriched; and (iii) several membrane-impermeant derivatives exist, allowing the selective labeling of cell-surface proteins. As a case study, we have used bovine serum albumin (BSA) as a soluble model protein to show that specific, experimentally induced, conformational changes result in a highly reproducible biotinylation pattern of lysine residues. Using this method, a comparison of the biotinylation patterns of band 3 allowed us to identify a distinct difference between iRBC and RBC.

EXPERIMENTAL PROCEDURES

Biotinylation of BSA and Carbonic Anhydrase—Fatty acid-free BSA (Roth, Karlsruhe, Germany) was dissolved in phosphate-buffered saline (PBS) to a final concentration of 10 mg/ml. For analysis of the biotinylation pattern of BSA, 1 ml of the solution (containing 1.4 × 10⁻⁴ mol of BSA) was biotinylated with 1-, 10-, 20-, 40-, and 60-fold molar eq (taking into account 60 lysine residues/BSA molecule) of sulfo-NHS-LC-biotin (Pierce) at 4 °C for 30 min. The reaction was stopped by the addition of glycine to a final concentration of 100 mM. The unreacted biotin derivative was removed from the sample by centrifugation at 3000 × g using a Millipore microconcentrator with a size exclusion of 5 kDa. This step was repeated twice after dilution with PBS before determining the protein concentration using the BCA assay (Pierce). For bovine carbonic anhydrase, 1.2 × 10⁻⁴ mol of carbonic anhydrase were biotinylated using a 40-fold molar excess of biotin derivative and processed as described for BSA. For analysis of the artificially induced conformational changes, BSA and carbonic anhydrase were dissolved in PBS as described above and subjected to an elevated temperature of either 56 or 80 °C for 30 min before cooling to room temperature and biotinylation with a 40-fold molar excess of biotin derivative. For affinity purification of the biotinylated proteins, 72 μl of streptavidin-Sepharose beads (Pierce) were washed extensively with PBS containing 1% (v/v) Nonidet P-40 before mixing the beads with 100 μg (1.44 × 10⁻⁶ mol) of biotinylated proteins. After overnight incubation at 4 °C, the unbound non-biotinylated protein fraction was obtained as a supernatant after centrifugation at 10,000 × g for 10 min. The beads containing bound biotinylated protein were washed consecutively in 10 mM Tris-HCl (pH 7.5), 0.2% Nonidet P-40, 2 mM EDTA, and 150 mM NaCl; in 10 mM Tris-HCl (pH 7.5), 0.2% Nonidet P-40, 2 mM EDTA, and 500 mM NaCl; and finally in 10 mM Tris-HCl (pH 7.5). The bound protein fraction was eluted from the beads by boiling in 500 μl of denaturing SDS-PAGE sample buffer and separated by 12% SDS-PAGE.

Biotinylation of Erythrocyte Membrane Proteins—RBC were obtained from blood group A⁺ donors and infected with the human malaria parasite P. falciparum using standard conditions. RBC and iRBC were cultured separately in RPMI 1640 medium in the presence of 10% human plasma under standard conditions (13). Erythrocytes infected with mature stage parasites were isolated from culture by plasmagel flotation, resulting in >90% iRBC (14). Biotinylation of intact erythrocytes (either infected or non-infected) was performed as described (4). Briefly, 2 × 10⁶ RBC or iRBC were washed with PBS (pH 7.6), incubated in the same buffer containing different concentrations of the biotin derivative (2, 1, and 0.5 mg/ml) for 1 h at 4 °C in the presence of 100 μM furosemide, and subsequently sedimented at 1300 × g for 5 min at 4 °C. To block and remove unreacted biotin derivative molecules, cells were washed three times with PBS (pH 7.6) containing 100 mM glycine and then with PBS. To enrich for membrane proteins, cells were resuspended in distilled water supplemented with a protease inhibitor mixture containing antipain, chymostatin, aprotinin, trypsin inhibitor, Na-EDTA, pepstatin, leupeptin, and elastatinal (each at a concentration of 1 μg/ml) and lysed by three cycles of freezing and thawing. The membrane fraction was sedimented by centrifugation at 18,000 × g for 20 min, solubilized in SDS-PAGE sample buffer, and separated by 7.5% SDS-PAGE.

Western Blot Analysis—Proteins separated by SDS-PAGE were transferred to nitrocellulose membranes using standard procedures. To detect biotin-labeled proteins, the membranes were blocked with 3% BSA in PBS (pH 7.4) for 1 h at room temperature before incubation for 20 min with alkaline phosphate-conjugated streptavidin (1:10,000/3% BSA in PBS (pH 7.4); Pharmingen). The membranes were washed three times with 10 mM Tris-HCl (pH 7.4) and 150 mM NaCl, and the biotin-labeled proteins were visualized after staining with nitro blue tetrazolium and 5-bromo-4-chloro-3-indolyl phosphate.

Sample Preparation for Mass Spectrometry (MS)—Protein bands were excised from polyacrylamide gels and subjected to in-gel trypsin digestion before MS analysis (15). Briefly, the excised spots were washed with 50% (v/v) acetonitrile in 200 mM ammonium bicarbonate, and the destained protein was in gel-reduced with 10 mM dithiothreitol in 100 mM ammonium bicarbonate for 1 h at 56 °C and alkylated with 50 mM iodoacetamide in the same buffer for 45 min at room temperature in darkness. The gel pieces were washed with 100 mM ammonium bicarbonate, dehydrated in acetonitrile, and dried. The gels were re-swollen in 15 μl of 40 mM ammonium bicarbonate containing 20 μg/ml sequencing-grade trypsin (Promega Corp.) for 45 min at 4 °C. Excess protease-containing solution was discarded, and 5 μl of 5 mM ammonium bicarbonate were added to keep the gel pieces wet during the 18-h proteolytic cleavage step at 37 °C. To extract the peptides, 15 μl of diffusion solution (10% acetonitrile and 1% trifluoroacetic acid) were added, and the samples were sonicated for 45 min at 37 °C. The soluble portion of the sample was evaporated to dryness. For MS measurements, the samples were redissolved in 15 μl of 0.1% (v/v) trifluoroacetic acid and 5% (v/v) acetonitrile in water. The peptides were purified and concentrated using ZipTip™ columns made from the reverse chromatography resins POROS R2 and Oligo R3 (Applied Biosystems). The bound peptides were washed with a solution of 0.5% formic acid and eluted from the ZipTip™ columns with 2 μl of 33% (v/v) acetonitrile and 0.1% trifluoroacetic acid saturated with α-cyano-4-hydroxycinnamic acid directly onto a matrix-assisted laser desorption ionization (MALDI) sample plate and air-dried before analysis in the mass spectrometer.

Alternatively, for affinity enrichment of biotinylated tryptic peptides, SDS-PAGE-separated proteins were transferred to
nitrocellulose membranes and visualized by Ponceau staining.
The stained membranes were cut into 2 × 2-mm square pieces,
thoughly destained with water, treated with 10 mM dithiothreitol
in 100 mM ammonium bicarbonate for 1 h at 56 °C, and
alkylated with 50 mM iodoacetamide in the same buffer for 45
min at room temperature in darkness. The nitrocellulose pieces
were washed with 100 mM ammonium bicarbonate and dried.
The membranes were incubated in a minimal volume of 40 mM
ammonium bicarbonate containing 20 μg/ml sequencing-
grade trypsin and incubated at 37 °C for 18 h. The tryptic pep-
tides were extracted from the membrane pieces twice using
20% acetonitrile in 1% trifluoroacetic acid, with sonication at
37 °C for 45 min. The eluted peptides were resuspended in 500
μl of 100 mM ammonium bicarbonate and incubated overnight
with 72 μl of washed streptavidin-Sepharose beads to affinity
purify the biotinylated peptides. The unbound non-biotiny-
lated peptide fraction was obtained as a supernatant after cen-
trifugation at 10,000 × g for 10 min. This fraction was evapo-
rated to dryness and resuspended in 0.1% trifluoroacetic acid
before processing for MS. The Sepharose beads containing
bound biotinylated peptides were washed three times with 100
mM ammonium bicarbonate and 1% octyl glucoside. The bound
peptides were eluted with 70% acetonitrile, 5% trifluoroacetic
acid, and 1 mM d-biotin in 100 mM ammonium bicarbonate at
room temperature for 2 h. The solution was evaporated to dry-
ness and resuspended in 0.1% trifluoroacetic acid before pro-
cessing for MS.

MS Analysis—MS was performed using a Bruker Daltonics
Ultraflex™ mass spectrometer equipped with a nitrogen laser
(337 nm laser, 3-ns pulse width, and 50-Hz repetition rate) and
panoramic mass range focusing (PANTM) technology and high
precision calibration (HPCTM) for high mass accuracy. For
analysis of intact protein, mass spectra were acquired after an
external calibration using the reference protein standards tryp-
sinogen, protein A, bovine albumin, protein A2+, and bovine
albumin2+ (protein calibration standard II, Bruker Daltonics).
Mass spectra were acquired in the linear positive mode with a
pulsed extraction using ~100 laser shots, and the masses were
assigned and processed using BioTools™ and flexAnalysis™
software (Bruker Daltonics). For the trypsin digests, peptide
mass fingerprint spectra were acquired in the reflection posi-
tive mode with a pulsed extraction using an average of 100 laser
shots. The spectra were acquired after an external calibration
using reference peptides (peptide mixture II, Bruker Daltonics).
The spectra were further internally calibrated using trypsin
autodigest peaks (842.5100 and 2211.1046 Da). Monoisotopic
masses were assigned and processed using BioTools™ and
flexAnalysis™ software before submission to the Mascot pro-
gram (www.matrixscience.com) for searches against the non-
redundant NCBI Database. The following variable modifica-
tions were used in the searches: methionine oxidation, lysine
sulfo-NHS-LC-biotin-labeled, pyroglutamic acid from Glu at
the N terminus, pyroglutamic acid from Gln, and a fixed cyste-
ine carbamidomethylation modification. To analyze the
effect of missed proteolytic cleavage of peptides on the identi-
fication of the proteins and biotinylated lysine residues, the
searches were done allowing complete cleavage (0 missed cleav-
age sites) and allowing one or two missed cleavage sites. A mass
accuracy of 50 ppm or better was used in all identifications.

Tandem mass spectrometry (MS/MS) analysis was done
using the LIFT™ mode to provide i, a, b, and y ions. The masses
of the fragmented ions were submitted to the Mascot program
for database searching using the following parameters: peptide
mass tolerance of 100 ppm, MS/MS tolerance of 0.7 Da, and two
missed cleavage sites. The variable and fixed modifications of
amino acid residues were used as described for the peptide mass
fingerprint analyses.

To analyze the spatial distribution of lysine residues across
the structures of BSA and carbonic anhydrase II, the crystal
structure of the latter enzyme (Protein Data Bank code 1V9E)
was examined. As no crystal structure of BSA has been depos-
ited in the Protein Data Bank, a homology model was obtained
from the SWISS-MODEL Repository (17) based on the crystal
structure of human serum albumin as a template (protein data
base searching using the following parameters: peptide
mass tolerance of 100 ppm, MS/MS tolerance of 0.7 Da, and two
missed cleavage sites. The variable and fixed modifications of
amino acid residues were used as described for the peptide mass
fingerprint analyses.

RESULTS AND DISCUSSION

Biotinylation of BSA Is Saturable but Incomplete—Sulfo-
NHS-LC-biotin derivatives possess an active ester group that
reacts with the primary amines of proteins and/or the ε-amino
group of lysine residues, thereby forming an amide bond (Fig.
1). For each lysine residue, this reaction results in an increase
in mass of 339.161 Da. If all 60 lysine residues of the BSA molecule
were biotinylated, the calculated mass shift of the protein would
be 20.34 kDa. BSA was biotinylated with increasing molar ratios of sulfo-NHS-LC-biotin to protein and analyzed by time-of-flight MS in linear mode. The mass of the protein increased in an almost linear correlation with the molar excess of the biotin derivative, up to a 40-fold molar excess of the derivative (Table 1). The maximal increase we observed was 6.1 kDa, accounting for ~18 biotinylated lysine residues. Thus, the biotinylation of lysine residues in the BSA molecule is incomplete. Analysis of the biotinylated protein by SDS-PAGE confirmed the increase in molecular mass of Coomassie Blue-stained protein, which was verified by biotin detection with streptavidin (supplemental Fig. 1).

To investigate whether biotinylation occurs randomly or whether specific lysine residues are preferentially labeled, biotinylated BSA was analyzed by peptide mass fingerprinting. Trypsin, the most commonly used protease to generate peptides for peptide mass fingerprinting, cleaves peptides at the C termini of the basic amino acid residues arginine and lysine. Because biotinylation occurs at the protonated ε-amino group of the lysine residue, the modification is likely to affect tryptic cleavage, leading to the generation of peptides containing a missed cleavage site. To analyze biotinylated peptides, BSA was first transferred to nitrocellulose filters and trypsinized. Subsequently, the peptides were affinity-purified and subjected to MS using MALDI. (21) revealed almost identical peptides, with only one additional peptide having two missed cleavage sites (supplemental Fig. 2). In the subsequent bioinformatics analyses, we therefore allowed for two missed cleavage sites. BSA biotinylated with different molar ratios of sulfo-NHS-LC-biotin was excised from polyacrylamide gels, cleaved with trypsin, and subjected to MS in the reflectron mode. Data base searches assuming complete cleavage were unable to identify BSA-derived peptides, indicating that the peptides contained cleavage sites that were not recognized by the protease. Allowing for one or two missed cleavage sites led to an increased number of identifiable peptides, with only one additional peptide having two missed cleavage sites (supplemental Fig. 2). In the subsequent bioinformatics analyses, we therefore allowed for two missed cleavage sites.

| Sulfo-LC-biotin/BSA molar ratio | Mass of BSA by MS Da | Approximate no. incorporated biotin residuesa | Sulfo-NHS-LC-biotin/BSA molar ratio |
|--------------------------------|---------------------|---------------------------------------------|-----------------------------------|
| 0                             | 66,525.3            | None                                        | 100                               |
| 1                             | 66,598.0            | 5                                           | 10                                |
| 10                            | 68,269.0            | 10                                          | 20                                |
| 60                            | 72,595.5            | 18                                          | 40                                |

*Each modified lysine residue resulted in an increase in mass of ~339 Da.*

The analysis of the intact protein, the correlation between the numbers of biotinylated lysines and biotin concentration was almost linear (Table 2). The numbers of the modified lysine residues identified by MS/MS correlated with the predicted numbers after MS of the intact protein in the linear mode (Table 1). More important, when biotin concentrations below the point of maximal incorporation were used, labeling of lysine residues did not occur randomly, but at specific sites. Residues that were biotinylated with lower concentrations of the derivative were also labeled when higher concentrations were used. These observations indicate a concentration-dependent, preferential accessibility of individual residues.

Recently, Huang et al. (21) used hydrophilic and hydrophobic cross-linkers and subsequent MS to probe the tertiary structure of BSA. One of the cross-linkers was bis(sulfo succinimidyl) suberate, which has a reaction mechanism similar to that of sulfo-NHS-LC-biotin. They identified a total of 20 modified lysine residues, 13 of which were also labeled with sulfo-NHS-LC-biotin in our experiments. Three of the modified residues (Lys<sup>138</sup>, Lys<sup>140</sup>, and Lys<sup>499</sup>) identified in the previous study were detected in all of our experiments, although they were non-biotinylated. One residue (Lys<sup>88</sup>) was not detected at all in our experiments, and one residue (Lys<sup>399</sup>) was detected only once in its biotinylated form. Two residues (Lys<sup>437</sup> and Lys<sup>523</sup>) were also biotinylated in our study, but only after exposure of BSA to high temperature (see below). We also identified five additional lysine residues (Lys<sup>36</sup>, Lys<sup>100</sup>, Lys<sup>266</sup>, Lys<sup>499</sup>, and Lys<sup>559</sup>) that had not been detected by Huang et al. (21), and it is therefore not possible to directly compare the labeling status in the two experiments.

In conclusion, different ionization conditions (electrospray versus MALDI) revealed almost identical peptides, and despite the use of different modifying reagents (bis(sulfo succinimidyl) suberate versus sulfo-NHS-LC-biotin), the majority of the modified residues were identical in both
studies, underscoring the reproducibility of the biotinylation reaction described above.

In light of a recent report (22) that lysine modification by N-succinimidyl propionate lacking the biotin moiety may induce conformational changes in a low molecular mass protein that is detectable by CD measurements, we investigated the possibility that the reaction between sulfo-NHS-LC-biotin and the lysine residues induces structural changes in the BSA molecule. CD spectroscopy was used to monitor changes in the structures of untreated BSA and BSA reacted with increasing molar ratios of the biotin derivative as described above. Far-UV (180–300 nm) measurements were performed at room temperature with a Jasco 810 spectrometer using 50 mM BSA in HEPES. The observed protein CD spectrum is an average of CD signals from all structures and reflects the geometric variability in the secondary structure. There was no significant difference in the spectra of untreated BSA and BSA reacted with the biotin derivative at different molar ratios (data not shown). In all the cases, the BSA structure is predominantly composed of α-helices, and the biotinylation reaction did not induce structural changes that were detectable by CD. In comparison with the protein of the light-harvesting complex that has been analyzed in a previous study (22) and that has a maximum of two disulfide bonds, the BSA molecule forms 17 intramolecular disulfide bonds (23). We anticipate that the structural compactness of BSA is the reason why this protein does not undergo a conformational change upon biotinylation.

To obtain some structural insight into the accessibility of the lysine residues of BSA, a homology model of BSA (NCBI accession number P02769) based on the crystal structure of human serum albumin as a template was consulted (sequence identity of 76%). All lysine residues of BSA were examined with respect to a possible correlation with the following structural descriptors: (i) spatial accessibility (visual inspection and calculation of the solvent-accessible surface), (ii) involvement in a hydrogen bond along with the associated energy of these hydrogen bonds, and (iii) electrostatic potential in the close environment of the considered residues. For descriptors i and iii, no clearly evident correlation could be discovered, whereas the involvement of a lysine residue in a hydrogen bond seems to significantly decrease the probability of biotinylation (supplemental Table 1). For BSA, only 7 of 24 lysine residues involved in a hydrogen bond reacted with sulfo-NHS-LC-biotin. However, it has to be taken into account that two of these seven lysines that do not fit the correlation are mutated in the homology model with respect to the crystallographically characterized template, thus increasing the uncertainty about their actual involvement in hydrogen bonds. To seek additional descriptors that might explain the deviating reactivity of the lysine residues involved in hydrogen bonding, we calculated the average energy of the hydrogen bonds separately for those lysines that become biotinylated and those that remain non-biotinylated. The mean energy for the non-biotinylated resi-
dues is more favorable by a factor of 3 than that for those that become biotinylated (excluding Lys100 and Lys245, which are mutated in the model with respect to the template). This rather pronounced discrimination based on the residue geometries found in the experimental structures suggests that those of the originally hydrogen-bonded lysines that are biotinylated have been involved in significantly weaker hydrogen bonds. Obviously, the participation in sufficiently strong hydrogen bonds prevents reaction with sulfo-NHS-LC-biotin, which is in accordance with previous notions that the involvement in hydrogen bonding may reduce the reactivity of lysine residues (22, 24).

**Elevated Temperatures Expose Novel Lysine Residues**—The highly reproducible biotinylation pattern of BSA and the obviously highly ordered accessibility of individual lysine residues prompted us to investigate whether treatments that result in conformational changes in the protein affect biotinylation patterns. BSA has a heart-shaped structure of three homologous domains that are divided into nine loops by 17 disulfide bonds (23). Heat-treated serum albumin undergoes a conformational change that is reversible as long as the temperature does not exceed 65 °C. The conformational change induced by higher temperatures is irreversible, but does not necessarily result in the complete destruction of the ordered structure (25). In our experiments, BSA was biotinylated at room temperature and after heating at 56 or 80 °C. For BSA treated at 56 °C, MS analysis revealed the identical biotinylated peptides as detected at room temperature. At 80 °C, two additional lysine residues (Lys437 and Lys523) were found biotinylated by MS/MS. As both of these residues were identified as non-biotinylated in the samples treated at the lower temperatures (Fig. 2, A and B), we conclude that heating, leading to an irreversible conformational change in the protein, allows access of biotin to these residues. Lys523 is in helix 2 of subdomain IIIb of BSA (23), and we suggest that this domain undergoes a considerable and irreversible conformational change upon heating to 80 °C. It is noteworthy that both Lys437 and Lys523 were identified as labeled residues in the study by Huang et al. (21), who performed the reaction at room temperature. Although the molecular masses of sulfo-NHS-LC-biotin and bis(sulfosuccinimidyl)
FIGURE 4. Differential biotinylation of lysine residues of band 3 in iRBC and RBC. Shown are enlarged sections of the MALDI mass spectra for band 3 from *P. falciparum*-infected erythrocytes and non-infected erythrocytes. In non-infected erythrocytes (RBC), Lys826, Lys829, and Lys892 in peptides with masses 1612.76, 1027.70, and 2864.19 Da, respectively, were biotinylated. In infected erythrocytes, Lys829 and Lys892 were biotinylated, but Lys826 was not. The arrow in A shows the absence of the biotinylated peptide in the spectra for band 3 from infected erythrocytes. a.u., arbitrary units. Intens., intensity.
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suberate are similar (556 and 572 Da, respectively), the lengths of the spacer arms differ by ~2-fold, which may explain the differential accessibility of these residues to the two compounds.

We also carried out a similar analysis on carbonic anhydrase, a 28-kDa single-domain protein with a dominating β-sheet that extends throughout the entire molecule (26). The thermal denaturation curve of carbonic anhydrase has a classical sigmoid shape with a melting temperature at 64.2 °C (27). Only minor structural changes, if any, are to be expected at this pre-transition temperature (27). Carbonic anhydrase has a total of 18 lysine residues. After biotinylation with a 40-fold molar excess of the biotin derivative at either room temperature or 56 °C, 15 lysine residues were detectable by MS, seven of which were biotinylated (supplemental Table 2). Heating the protein to 80 °C and cooling to room temperature prior to biotinylation had no effect on this pattern because of the rapid refolding of the protein.

Also in the case of bovine carbonic anhydrase II, we examined the available crystal structure for the putative accessibility of the lysine residues. The same descriptors as described above were studied. Similarly, analysis suggested a correlation between the involvement in hydrogen bonding and the decreased probability of biotinylation (supplemental Table 3). Only one of eight lysine residues involved in a hydrogen bond is biotinylated.

As shown for BSA, heating the protein increased the number of biotinylatable lysine residues without the loss of biotinylating sites accessible at low temperature. Presumably, the higher temperature resulted in a generally more open, possibly partially unfolded or denatured structure of the BSA molecule. This observation is supported by our CD spectrometry measurements showing a change in the conformation of BSA with a shift from a predominantly α-helix structure to the more open β-sheet conformation after exposure to an elevated temperature of 80 °C. However, we anticipate that conformational changes, others than those induced by elevated temperature, do not necessarily result in a gain of biotinylatable sites. Depending on the experimental circumstances, a loss of such sites, e.g. as a result of steric hindrance, is equally possible.

Comparison of the Biotinylation Patterns of Band 3 in RBC and iRBC—Biotinylation of BSA revealed a linear correlation between the molar ratio of the biotin derivative and biotinylated lysine residues until all sufficiently reactive residues were saturated. The band 3 protein is present on the erythrocyte surface at 10^6 copies/cell (28). To ensure excess of the biotin derivative, standardized numbers of either infected or non-infected erythrocytes containing band 3 were biotinylated using concentrations of sulfo-NHS-LC-biotin ranging from 0.5 to 2 mg/ml. The reactions were carried out in the presence of furosemide to minimize internal labeling of infected erythrocytes (4). Proteins contained in membrane fractions of erythrocytes were separated by SDS-PAGE and stained with Coomassie Blue, and the band corresponding to band 3 was excised and analyzed. As described above, in the data base searches, two missed cleavage sites per peptide were allowed. Within the chosen range, the concentration of the biotin derivative did not have an effect on the biotinylation status of individual lysine residues (data not shown).

Band 3 is a multimembrane-spanning protein with 11–14 transmembrane domains (16, 29). According to the predicted topology, five domains are exposed extracellularly. Whereas three domains are composed of fewer than 10 amino acid residues, two domains (Phe^{543}_–Pro^{568} and Glu^{626}_–Met^{663}) are composed of 26 and 38 amino acids, respectively. One additional domain (Gly^{802}_–Leu^{835}) is predicted to exhibit enhanced flexibility, which can vary between an extracellular and intracellular topology. Although the various models differ in details, they are consistent in that, of the total 29 lysine residues, nine residues reside in the extracellular loops, four of which are located in one of two large loops. Four additional lysine residues are found in the flexible loop.

After surface biotinylation of RBC, three lysine residues were found to be biotinylated (Fig. 3). Two of these residues (Lys^826 and Lys^829) are located in the prominent flexible loop, and one (Lys^892) in a predicted intracellular domain. In contrast, in iRBC, only two lysine residues (Lys^829 and Lys^892) were biotinylated. Although it is conceivable that conformational changes take place only in a certain percentage of the band 3 molecules, we were unable to detect biotinylated Lys^826 in iRBC, and conversely, peptides from RBC containing Lys^826 were always biotinylated (Fig. 4, A–C). These results suggest that all band 3 molecules undergo a conformational change in iRBC. We have no explanation why Lys^892, which is predicted to be intracellular, was found biotinylated in both samples. The overall low sequence coverage for band 3 (30%) can be attributed to the high proportion of hydrophobic domains present in this molecule.

In conclusion, biotinylation of proteins is highly reproducible and appears to be dependent on protein conformation. Possibly the involvement of the ε-amino group of lysine in hydrogen bonding provides an explanation for the gradual reactivity observed for the various residues. Thus, conformational changes or alternative structural transformations in proteins lead to alternative biotinylation patterns. As shown for the band 3 molecule in non-infected and malaria parasite-infected erythrocytes, this approach allowed us to identify a differentially biotinylated lysine residue. This residue is positioned in the extracellular loop in a region that is also differentially recognized in non-infected and infected erythrocytes by a monoclonal antibody (8). We suggest that surface biotinylation of intact cells followed by MS is a rapid method for identifying putative conformational changes in surface proteins, for example, following pathogen infection or under varying physiological conditions.

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