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Letters to the Editor

Measurement of Exofacially Reactive Lysines on Human Erythrocyte Band 3 Using Pyridoxal 5′-Phosphate

I read with interest the recent paper by Bar-Noy and Cabantchik [1] on pyridoxal 5′-phosphate (PLP) labeling of band 3. Their findings generally confirm our previous results [4] showing: (i) that *both* integral chymotryptic subdomains of band 3 (CH17 and CH35) have lysines which are "sensitive" to the DNDS (4,4′-dinitrostilbene-2,2′-disulfonate)-induced conformational change [*see* 1, *Note Added in Proof*) and (ii) that exclusive (but partial) coverage of CH17 by PLP significantly inhibits anion transport under "symmetric" labeling conditions in chloride (*see* Fig. 1 of 1, $Cl_i - Cl_o$ + PLP + DNDS *versus* control). Yet, despite this general agreement, the authors state that their total stoichiometry was 2 moles of PLP per mole of band 3, which is about one-half of what we have observed at saturating PLP. In addition, the authors suggested that our observation of an increase in PLP labeling associated with osmotic hemolysis and resealing [5], was somehow due to contamination by unsealed ghosts.

Considering the last point first, we showed controls indicating that our resealing methods yield no detectable unsealed or leaky ghosts [5]. Osmotic hemolysis and resealing had two effects on intact red cells prelabeled with PLP. First, there was a partial "deinhibition" of anion exchange which was *not* observed in cells prelabeled by covalent binding of DIDS (4,4′-diisothiocyano-2,2′-disulfonate). This difference shows that deinhibition is not trivially related to the cell manipulation step, but rather is band 3 specific. Leaky ghosts are very readily detectable using our continuous monitor, stopped-flow transport assay method [5], and no evidence suggesting that the ghosts were leaky to anions could be found. Second, we clearly showed that increased PLP labeling *only* occurred on the CH17 subdomain of the protein, despite the fact that CH35 has nearly four times the number of lysines compared to CH17 [7]. If general contamination by unsealed ghosts was the cause of our effect, one would have expected to see increased PLP labeling on both subdomains of band 3.

We believe that the difference in whole molecule PLP labeling stoichiometry between our study and the study published in this journal [1], arises from a substantial difference in labeling conditions. We added excess borohydride to mixtures of PLP and intact cells without significant dilution (<2%). We then *isolated* the protein, or we investigated the distribution of the label on alkali-stripped, chymotrypsinized membranes where peripheral proteins are removed and where PLP labeling of glycophorin is eliminated [1, 4]. In contrast, Bar-Noy and Cabantchik [1] state that they added excess lysine (50 mM) and then washed their cells twice before adding the fixative. As is well known [3], protein lysine residues are readily derivatized by PLP *via* Schiff's base formation. Equally well known is the fact that the Schiff's base can be readily hydrolyzed [3]. In adding excess lysine and washing, the authors assume that all of the derivatized lysines have stable Schiff's base complexes. Although washing at pH 8 may minimize hydrolysis of the Schiff's base linkage, evidence to establish whether such conditions eliminate hydrolysis of *all* PLP molecules initially bound to band 3, was not given. In our experience, washing prior to fixation reduces the total amount of band 3 labeling [4]. Thus, it is not surprising that Bar-Noy and Cabantchik [1] found a lower stoichiometry. Addition of an excess of competitor for the ligand should be expected to shift the equilibrium of easily hydrolyzable, band 3-bound PLP molecules toward the dissociated state. Washing in the presence of lysine should further promote dissociation, leaving the less easily hydrolyzable PLP molecules bound. Thus, if one wishes to count all of the exofacially accessible lysines, it would seem necessary not to add a competitor and not to wash the cells prior to fixation.

We found about 4 moles of PLP bound per mole of band 3 *at saturation* of exofacial sites in the *absence* of DNDS (2 moles of PLP per mole of each integral chymotryptic subdomain). Recent sequence data show 4 CH17 lysine residues and 17 CH35 lysine residues on the integral domain of band 3 [7]. We also showed that addition of DNDS *increased* PLP labeling of CH17. In further studies described in the *Note Added in Proof,* Bar-Noy and Cabantchik [1] state that they also see increased CH17 labeling with addition of DNDS. This effect may be ascribed to the "recruitment"-related conformational change expected when an impermeant ligand like DNDS is added to the outer surface of band 3 [6]. But that conformational change may need to be of *global* proportion to explain how nonmutually competitive CH17 lysines can be affected by DNDS binding to CH35 [4]. Observation of a similar site recruitment effect at the apparently competitive lysines on CH35, requires that PLP titration experiments be performed at constant DNDS, so as to allow PLP to displace reversibly bound DNDS at high PLP concentrations. In our experiments, extrapolation to saturation, of the PLP labeling curve for CH35, showed a DNDS-induced increase in PLP labeling which was comparable to that seen for CH17 [4]. Thus, CH35 responds to the stilbene-mediated conformational change in an apparent (but not proven) competitive fashion, while CH17 responds in a conformationally coupled fashion. Evidence favoring a large protein conformational change in band 3, was given in recent measurements of the activation volume associated with human erythrocyte anion exchange [2]. New protein crosslinking evidence from our laboratory [6] indicates that ligand-mediated conformational changes can occur at the level of protein quaternary structure for band 3 [6]. This offers a classical explanation for both the large activation volume observed [2] and for the overwhelming number of papers demonstrating allosteric effects on band 3 [6].

In summary, while further work is necessary to specifically identify the exofacial lysine residues on band 3 which are capable of being labeled by PLP, no agreement on the actual number of

such lysines should be expected if different fixation procedures are used.

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Response to: Measurement of Exofacially Reactive Lysines on Human Erythrocyte Band 3 Using Pyridoxal 5′-Phosphate

Dr. Salhany's comments that the ostensible differences in the stoichiometry of PLP-NaBH binding to band 3 polypeptides found in Bar-Noy and Cabantchik's work [1] as compared to those found in his studies [8, 9] might have resulted from differences in labelling conditions of cells. While our recent studies are similar methodologically and quantitatively to those of Hamasaki's group [6, 7], the analogous studies by Salhany and coworkers resemble more the original studies by Cabantchik et al. [4, 5]. The main differences seem to stem from the fact that the irreversible reduction of PLP-membrane Schiff's bases with NaBH, following initial interaction between PLP and cells (or ghosts) was carried out: after washing with lysine [1, 6] or after resuspension of cells in buffered medium [4, 5] as compared with no washing but with excess reduction agent [8, 9].

It is our view that irrespective of the method used, the critical point is to estimate the degree of inhibition attained with a given number of PLP molecules irreversibly attached per cell in general and with band 3 in particular. We have found that after formation of the Schiff bases at the membrane surface and washing the cells with ice-cold buffer (±50 mM lysine), more than 90% of the inhibitory effect on anion transport is retained (as compared with no washing), while incubating the cells with 50 mM lysine for 5 min at 37°C reduces the inhibitor's effect only by 50% (*unpublished observations*). This clearly indicates that the majority of Schiff's bases formed between PLP and functionally relevant lysines are relatively stable (other examples of stable PLP-Schiff bases are those formed between PLP and hemoglobin [2, 3]. In our conditions of cell labelling with PLP-NaBH (80% inhibition of transport), the number of probes found per ghosts derived from labelled cells is less than 2×10^6 and more than 80% are on band 3 (as shown also in ref. 6). The possibility that a small fraction of Schiff's bases (unreduced by borohydride) are reversed upon exposure of the outer membrane to hemoglobin (which has a high affinity for PLP) during cell lysis was considered, and it was found more than 90% of the inhibitory effect elicited by PLP-$NaBH_4$ in cells was retained in ghosts. In the analogous study carried out by Salhany and coworkers it was shown that PLP (at concentrations which cause 90% inhibition) labels 3 lys per polypeptide in the band 3 zone of the gel [8, 9]. After preparation of ghosts new sites (exofacial?) for PLP labelling were detected [8].

In our recent study we adopted Nanry et al.'s [7] method (washing with lys) and not our original procedure (washing with cold buffer) so as to minimize labelling of components other than band 3 or components which comigrate with band 3 polypeptides (primarily glycophorins). It is not clear to us, that in the conditions used by Salhany [8, 9], all the PLP-cell membrane associated probes which become irreversibly fixed with $NaBH_4$ are associated exclusively with band 3 or proteolytic fragments derived from band 3, as it was shown previously to be the case [4, 5].

For the case of surface labelling of resealed ghosts, the possibility that a (small) fraction of ghosts which remain unsealed will be "highly and nonspecifically" labelled is considerably greater than detection of unsealed ghosts by transport assays which are "highly biased" for sealed compartments.

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