Role of Band 3 Tyrosine Phosphorylation in the Regulation of Erythrocyte Glycolysis*

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Previous studies demonstrated that the *in vitro* tyrosine phosphorylation of the human erythrocyte anion transporter, band 3, prevented the binding of various glycolytic enzymes to the N terminus of the cytoplasmic tail. Since these enzymes are inhibited in their bound state, the functional consequences of band 3 tyrosine phosphorylation in the red cell should be to activate the enzymes and elevate glycolysis. We searched for various enhancers of band 3 tyrosine phosphorylation using a novel assay designed to measure the phosphotyrosine levels at the band 3 tyrosine phosphorylation/glycolytic enzyme-binding site. This assay measures the extent of phosphorylation of a synthetic band 3 peptide entrapped within resealed red cells. Using this assay, three distinct compounds, all mild oxidants, were found to stimulate the tyrosine phosphorylation of band 3. All three compounds were also found to elevate glycolytic rates in intact erythrocytes. Moreover, the antitumor drug adriamycin was found to coordinately prevent these agents from stimulating both band 3 tyrosine phosphorylation and erythrocyte glycolysis. These results suggest a possible function for a protein tyrosine kinase in human erythrocytes, to regulate glycolysis through the tyrosine phosphorylation of band 3.

Although it has been a decade since the original discovery of phosphotyrosine and the accompanying protein tyrosine kinases (PTKs)[1], the actual pathways through which these enzymes participate in cell growth and division have yet to be defined. One of the most intriguing aspects of the cellular PTKs is their presence in terminally differentiated cells such as platelets (1, 2), neurons (3), and erythrocytes (1, 4). The high amount of PTK activity found in red cells and platelets is especially curious since both cell types lack a nucleus and therefore undergo neither division nor differentiation. We began our studies of the PTKs in human erythrocytes with the goal of deciphering the functional consequences of their presence in this relatively well-defined cell.

One of the major *in situ* substrates for the human erythrocyte PTK is the transmembrane anion transporter, band 3 (4, 5). The tyrosine phosphorylation site has been identified as tyrosine residue 8 and probably tyrosine 21 at the extreme N terminus of the cytoplasmic tail of band 3 (4, 6) (for a review of the structure of band 3 see Ref. 7). Although not thought to be involved with the anion transport function of band 3, sequences surrounding these tyrosines have been associated with the intracellular binding of several cytosolic proteins, including the glycolytic enzymes aldolase, phosphofructokinase and glyceraldehyde-3-phosphate dehydrogenase (G3PDH) (8–13). Of these, the binding of aldolase and G3PDH have been the most thoroughly characterized.

In a previous publication (6), we reasoned that one of the functional consequences of the phosphorylation of tyrosines 8 and 21 might be to alter the binding of the glycolytic enzymes to band 3. We tested this hypothesis and reported that *in vitro*, the phosphorylation of band 3 on N-terminal tyrosine residues prevented the binding of G3PDH, aldolase, and phosphofructokinase (6). Since these enzymes have been shown to be inhibited in their bound state (9, 14), one predicted consequence of N-terminal band 3 tyrosine phosphorylation would be an enhanced rate of glycolysis in the intact cell. We tested this hypothesis and report here that in whole erythrocytes, extracellular agents that stimulate tyrosine phosphorylation of band 3 also enhance glycolysis. Additionally, we observe that the antitumor drug, adriamycin, coordinately blocks both the increase in band 3 tyrosine phosphorylation and the stimulation of glycolysis.

MATERIALS AND METHODS

Glycolysis Assays—Human blood was purchased from the Central Indiana Regional Blood Bank and used before its expiration date. Blood was centrifuged at $3,000 \times g$, 4 °C, for 5 min, the buffy coat removed, and the packed cells resuspended in Ringer's buffer (125 mM NaCl₂, 3 mM KCl, 1 mM MgCl₂, 1.2 mM Na₂HPO₄, 5 mM glucose, 30 mM Hepes, pH 7.4), and washed three times. Washed cells were preincubated at 50% hematocrit in Ringer's buffer at 37 °C for 1 h. Glycolysis assays were performed by incubating intact red cells (2 ml of 50% hematocrit in Ringer's buffer) in the presence or absence of various exogenous agents at the indicated concentrations for 10 min. The incubations were stopped with 4 ml of ice-cold 1 M HClO₄ and neutralized with K₂CO₃. The concentrations of various glycolytic intermediates as well as lactate were determined according to classic procedures (15). All assays were repeated several times, in duplicate. Glycolysis assays were also performed on lysed red blood cells resealed by the procedure described below (data presented in Fig. 3) with similar results to those obtained with whole red cells.

Phosphorylation of the Band 3 Peptide Resealed in Red Cells—For the quantitative measurement of band 3 PTK activity, human blood was initially centrifuged at $3,000 \times g$, 4 °C, for 5 min. After the buffy coat had been removed, packed cells were resuspended in 25 mM Hepes, pH 7.2, 100 mM KCl, 100 mM sucrose, and 5 mM MgCl₂ (washing buffer), and washed three times. After washing, the eryth-

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[1] The abbreviations used are: PTK, protein tyrosine kinase; G3PDH, glyceraldehyde-3-phosphate dehydrogenase; SDS-PAGE, sodium dodecyl sulfate-polyacrylamide gel electrophoresis; Hepes, 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid; PTPase, protein tyrosine phosphatase.
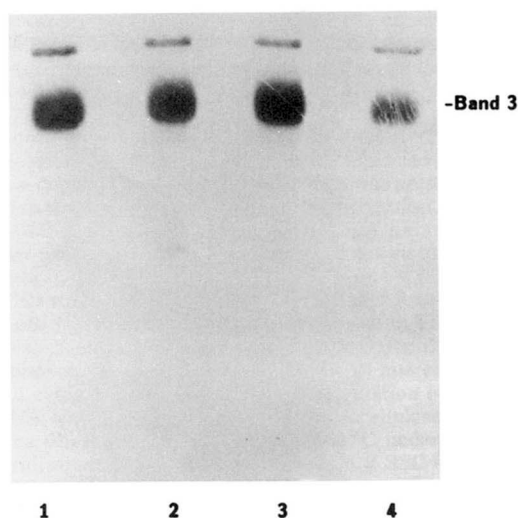
rocytes were diluted to 90% hematocrit with washing buffer. 80 μl of packed cells were then mixed with 10 μl of 500 μM [γ-^{32}P]ATP (Du Pont-New England Nuclear, specific activity, 200–2,000 cpm/pmol) and either 10 μl of water or 10 μl of 10 mM band 3 peptide (synthesized by the Purdue University Peptide Synthesis Facility). The band 3 peptide corresponding to residues 1–15 of band 3 according to Kaul *et al.* (10) has the following sequence: Met-Glu-Glu-Leu-Gln-Asp-Asp-Tyr-Glu-Asp-Asp-Met-Glu-Glu-Asn. The samples in 1.5-ml Eppendorf tubes were placed at −20 °C in a prechilled polystyrene test tube rack for 5 min to achieve mild freezing. The samples were then removed and thawed on ice and incubated at 37 °C for 15 min to facilitate resealing. Essentially all the cells were lysed by the mild freeze-thaw procedure and approximately 15% of the erythrocytes successfully resealed as determined by hematocrit measurements and the retention of both rhodamine- and ^{125}I-labeled peptide after repeated washings. Peptide incorporation into resealed cells was assessed by fluorescence microscopy using rhodamine-β-isothiocyanate (Sigma)-labeled band 3 peptide and ^{125}I-labeled band 3 peptide. Fluorescence microscopy revealed that approximately 90% of the resealed cells contained peptide. The intracellular peptide concentration was estimated at approximately 0.5 mmol/liter of cell water using the radiolabeled peptide.[2] The resealed samples were centrifuged at 2,250 × g, 4 °C for 5 min, and the packed cells were resuspended in washing buffer. This procedure was repeated three times in order to wash the resealed cells and remove any free membranes. The washed cells were resuspended in washing buffer and incubated for 10 min at 37 °C in the presence of various exogenous agents at the indicated concentrations. The resealed cells were then placed on ice, and 100 μl of 25% ice-cold trichloroacetic acid was added to lyse the cells and precipitate cellular protein. The samples were centrifuged, and the phosphorylated band 3 peptide was isolated from the supernatants following the procedure of Braun *et al.* (16).

Phosphorylation of Native Band 3 in Resealed Red Cells—Red cells were resealed in the presence of [γ-^{32}P]ATP, but in the absence of peptide, and stimulated with the various oxidants as described above. The cells were lysed in 1 ml of ice-cold hypotonic lysis solution (5 mM p-nitrophenylphosphate, 3 mM Na$_3$VO$_4$, 40 μg/ml leupeptin, 25 μg/ml aprotinin, and 20 μg/ml phenylmethylsulfonyl fluoride, and the lysates were centrifuged at 13,000 × g, 4 °C, for 2 min. The resulting membranes were dissolved in SDS sample buffer and analyzed by SDS-PAGE and autoradiography.

Partial Purification of the Band 3 Tyrosine Kinase—The band 3 tyrosine kinase was partially purified from human red cells using the procedure of Mohamed and Steck (17). 0.5% Triton X-100 insoluble skeletons were pelleted and resuspended in 25 mM Hepes, pH 7.0, 0.3% Triton X-100, 10 mM dithiothreitol, and 250 mM NaCl. The suspension was incubated on ice for 30 min and centrifuged at 60,000 × g for 15 min, and the supernatant was dialyzed against the same buffer lacking NaCl. This preparation contains both the kinase and some endogenous band 3. The cytoplasmic domain of band 3 was purified as previously described (18).

RESULTS

We have previously reported that *in vitro*, the phosphorylation of band 3 on tyrosine residues at the polypeptide's N terminus can block the binding and subsequent inhibition of glycolytic enzymes (6). To determine whether glycolysis might be affected by a similar tyrosine phosphorylation event *in vivo*, we examined enhancers of band 3 tyrosine phosphorylation for their effect on glycolysis in intact red cells. To identify such enhancers, red cells were lysed and resealed in the presence of [γ-^{32}P]ATP. The resealed cells were then stimulated by the addition of various extracellular reagents, and native band 3 phosphorylation was assessed by SDS-PAGE and autoradiography. Because mild oxidants had been reported to inhibit protein tyrosine phosphatases (PTPases) (19–22), we initially examined whether such compounds might elevate the level of band 3 tyrosine phosphorylation. Fig. 1 shows that the addition of the oxidants ferricyanide, diamide, and hydrogen peroxide to resealed red cells caused an increase in the phosphorylation of native band 3. The alkali stability

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[2] M. L. Harrison, C. Isaacson, P. Rathinavelu, R. L. Geahlen, and P. S. Low, manuscript in preparation.

FIG. 1. **Native band 3 phosphorylation in resealed red cells.** Autoradiogram of an SDS-polyacrylamide gel after alkali treatment (1 M KOH, heated to 55 °C for 2 h). Red cells were lysed and resealed in the presence of [γ-^{32}P]ATP. Washed, resealed cells were incubated with diamide (*lane 1*), hydrogen peroxide (*lane 2*), or ferricyanide (*lane 3*). Control cells (*lane 4*) were incubated in the absence of oxidants. Cells were disrupted in hypotonic lysis solution and the resulting membrane proteins separated by SDS-PAGE.

of the phosphorylation indicated the presence of phosphotyrosine which was confirmed by phosphoamino acid analysis (data not shown).

In order to obtain a more quantitative measure of band 3 tyrosine phosphorylation, as well as to directly determine the extent of tyrosine phosphorylation of the glycolytic enzyme-binding site on band 3, we developed a tyrosine phosphorylation assay specific for tyrosine 8 on band 3. This assay eliminated the need for continued phosphoamino acid analysis as well as provided for a measure of band 3 tyrosine kinase activity that was directed toward the tyrosine residue found in the glycolytic enzyme-binding site on band 3. The assay is based on the ability of a synthetic peptide entrapped within red cells to serve as an extractable substrate for the band 3 tyrosine kinase (see "Materials and Methods"). The amino acid sequence of the synthetic band 3 peptide corresponds to the tyrosine phosphorylation/glycolytic enzyme-binding site on band 3 and contains no serine or threonine residues. Thus, this assay has the distinct advantage of measuring the tyrosine phosphorylation of band 3 only at the glycolytic enzyme-binding site.

It was important to determine whether the synthetic pentadecapeptide would serve as a substrate for the band 3 tyrosine kinase. For this purpose, the kinase was partially purified from red blood cells and incubated with the band 3 peptide in the presence of [γ-^{32}P]ATP. As shown in Table I, the peptide became phosphorylated in the presence of the band 3 kinase. The effect of peptide on the phosphorylation of band 3 by the partially purified red cell PTK was also examined. The band 3 kinase preparation that contained both the kinase and some native band 3 was phosphorylated with [γ-^{32}P]ATP in the presence and absence of the synthetic peptide. As can be seen in Fig. 2A, the kinase readily phosphorylated the native band 3 protein (*lane 1*), as well as a purified preparation of the cytoplasmic domain of band 3 (*lane 2*), which contains the N-terminal glycolytic enzyme-binding site. Importantly, the phosphorylation of both intact band 3 as well as the cytoplasmic domain was significantly diminished in the presence of the band 3 peptide (*lane 3*). The alkali stability of the band 3 phosphorylation (Fig. 2B)
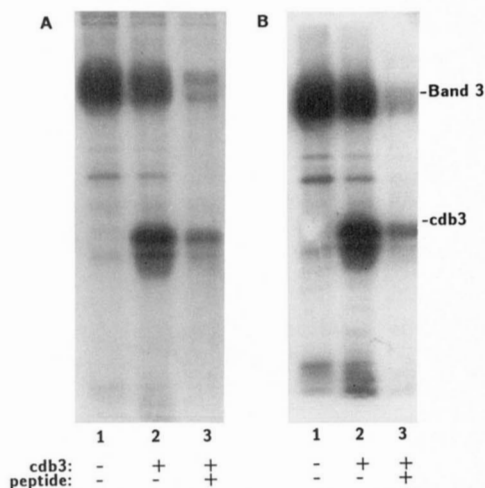
TABLE I

Phosphorylation of the band 3 peptide by a partially purified preparation of the red cell PTK

The synthetic band 3 peptide (3.5 mM) was incubated with a partially purified preparation of the red cell PTK in the presence of $[\gamma\text{-}^{32}P]$ATP (20 μM, 1.67×10^9 cpm/μmol), MnCl$_2$ (10 mM), and *p*-nitrophenylphosphate (5 mM). At the indicated times, the peptide was extracted and analyzed for radioactive content as described (16).

Time	Peptide phosphorylation
min	*cpm*[a]
15	20,826
30	49,592
60	72,392

[a] Background levels of radioactivity determined in the absence of peptide were 4,180 cpm after 15 min and 3,434 cpm after 60 min.

FIG. 2. **Inhibition of band 3 phosphorylation by the band 3 peptide.** A partially purified preparation of the band 3 tyrosine kinase containing native band 3 was phosphorylated in the absence (*lane 1*) or presence of purified cytoplasmic domain of band 3 (*cdb3*), 3.5 μM (*lanes 2* and *3*) and band 3 peptide, 1.2 mM (*lane 3*). The phosphorylation reactions were carried out in a total volume of 50 μl and contained 30 μl of the partially purified kinase (1.5 mg/ml), 10 mM MnCl$_2$, 5 mM *p*-nitrophenylphosphate, and 5 μl $[\gamma\text{-}^{32}P]$ATP (5 μCi/mM). The reactions were for 10 min at 37 °C. The reactions were terminated by the addition of SDS sample buffer. The samples were analyzed by SDS-PAGE on 10% gels and processed for autoradiography. *A*, autoradiogram of gel before alkali treatment and *B*, autoradiogram of alkali treated (1 M KOH, 2 h, 55 °C) gel. The position of the endogenous band 3 and the added cytoplasmic domain of band 3 are indicated.

indicated the presence of phosphotyrosine which was confirmed by direct phosphoamino acid analysis of the band 3 protein (data not shown). These results demonstrated that the synthetic band 3 peptide would serve as a substrate for the red cell band 3 PTK.

The synthetic band 3 peptide, along with $[\gamma\text{-}^{32}P]$ATP, was resealed into red blood cells and the washed resealed cells were treated with the mild oxidants described above. The oxidant ferricyanide $(Fe(CN)_6^{-3})$ was selected as an initial candidate since it has been reported to increase glycolysis in red cells (23, 24). As shown in Table II, when resealed red cells containing the band 3 peptide were exposed to extracellular ferricyanide, peptide phosphorylation was stimulated approximately 2-fold. Although ferricyanide is impermeable to red cells, it has been reported to oxidize intracellular redox substrates, most probably through an endogenous transmembrane redox system (25–27). We have observed that in isolated red cell membranes ferricyanide actually inhibits the *in vitro* phosphorylation of band 3 (data not shown) consistent with

TABLE II

Effect of ferricyanide and adriamycin on red blood cell glycolysis and band 3 peptide phosphorylation

Lactate production was measured in washed red blood cells after exposure to ferricyanide and/or adriamycin as described under "Materials and Methods." Band 3 peptide phosphorylation was measured in resealed red blood cells containing $[\gamma\text{-}^{32}P]$ATP and band 3 peptide after exposure to ferricyanide and/or adriamycin as described under "Materials and Methods."

	Lactate production	Peptide phosphorylation
	mM[a]	*cpm*[b]
Control	20.64	28,564
	23.95	26,129
Adriamycin (0.1 μM)	23.95	ND[c]
	23.20	
Ferricyanide (2 mM)	38.45	66,469
	39.75	67,616
Ferricyanide (2 mM) +	20.82	27,901
adriamycin (0.1 μM)	20.26	28,840

[a] Concentration/liter of packed cells.
[b] Background levels determined in cells resealed in the absence of the band 3 peptide averaged 2,718 cpm.
[c] ND, not determined.

the notion that ferricyanide must act extracellularly. Our data would suggest that one result of activating this transmembrane redox pathway in intact erythrocytes is either the activation of the band 3 tyrosine kinase or inhibition of the band 3 PTPase. As expected, exposure of intact (*i.e.* not resealed) red cells to $Fe(CN)_6^{-3}$ enhanced glucose metabolism, leading to a near doubling of lactate production during the 10-min incubation period (Table II). When mouse (a species whose band 3 lacks a binding site for the glycolytic enzymes) red cells were used, no stimulation of glycolysis was observed (data not shown). These initial results supported our hypothesis that in human red cells an increase in band 3 tyrosine phosphorylation leads to an increase in glycolytic rate.

To more critically evaluate the correlation between band 3 tyrosine phosphorylation and enhanced glycolysis, the stimulation of both processes by increasing concentrations of ferricyanide was compared under identical conditions. For this experiment glycolysis was measured in red cells that had undergone the identical resealing process as cells used in the phosphorylation assay except that peptide and ATP were omitted from the freezing solution. As illustrated in Fig. 3, the concentration curves generated for the two processes were nearly superimposable, strongly supporting the idea that the two events are causally related.

To learn whether the observed association between band 3 tyrosine phosphorylation and glycolytic flux might extend to other compounds, studies analogous to those with ferricyanide were also conducted with 1 mM hydrogen peroxide (Table III) and 25 μM diamide (Table IV). As seen in the tables, both compounds stimulated band 3 peptide phosphorylation and caused a simultaneous increase in glycolysis.[3] Since both diamide and hydrogen peroxide can penetrate the red cell membrane, their exact location of action is unknown, but like ferricyanide they appear to either increase band 3 PTK activity or inhibit the corresponding PTPase activity. We have observed that the PTPase inhibitor, orthovanadate, also

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[3] It should be noted that the data in Tables III and IV were generated on the same day using the same blood pack, while the data in Table II were generated earlier using a different blood pack. We have observed that basal lactate production can differ significantly among various blood samples.
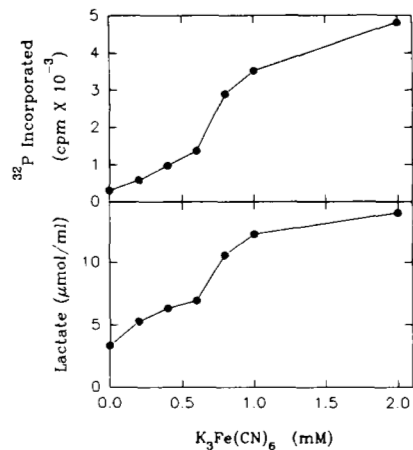
FIG. 3. **Stimulation of band 3 peptide phosphorylation and erythrocyte glycolysis as a function of $Fe(CN)_6^{-3}$ concentration.** *Top*, band 3 peptide phosphorylation was determined in resealed cells after incubation with 2 mM $Fe(CN)_6^{-3}$ as described under "Materials and Methods." The data represent the average of duplicate samples. *Bottom*, lactate content was determined in erythrocytes that had been resealed under conditions identical to those described for the band 3 peptide phosphorylation assay except that the resealing buffer lacked band 3 peptide and $[\gamma\text{-}^{32}P]$ATP. Following resealing and stimulation with 2 mM $Fe(CN)_6^{-3}$, the samples were processed for lactate content as described under "Materials and Methods." The data represent the average of duplicate samples.

TABLE III

Effect of diamide and adriamycin on red blood cell glycolysis and band 3 peptide phosphorylation

Lactate production was measured in washed red blood cells after exposure to diamide and/or adriamycin as described under "Materials and Methods." Band 3 peptide phosphorylation was measured in resealed red blood cells containing $[\gamma\text{-}^{32}P]$ATP and band 3 peptide after exposure to diamide and/or adriamycin as described under "Materials and Methods."

	Lactate production	Peptide phosphorylation
	mM^a	cpm^b
Control	7.33	7,886
	7.62	9,350
Diamide (25 μM)	12.87	20,107
	10.12	17,258
Diamide (25 μM) + adriamycin (0.1 μM)	6.32	6,787
	4.54	7,556

[a] Concentration/liter of packed cells.
[b] Background levels determined in cells resealed in the absence of the band 3 peptide averaged 618 cpm.

causes a simultaneous increase in band 3 peptide phosphorylation and erythrocyte glycolysis (data not shown).

Because of the possibility that the apparent relationship between band 3 tyrosine phosphorylation and altered glycolysis might simply be coincidental, we wished to test the effect of an inhibitor of tyrosine phosphorylation in our assay systems. The antitumor drug adriamycin was used for this purpose, since it had been reported to inhibit PTKs (28) as well as block the red cell transplasma membrane redox system through which ferricyanide has been reported to function (27, 29, 30). The effect of 0.1 μM adriamycin on lactate production and peptide phosphorylation was examined. As seen in Tables II–IV, adriamycin simultaneously inhibited the stimulation of both band 3 peptide phosphorylation and glycolysis caused by the three distinct redox reagents we have tested. Whether adriamycin's inhibitory effect was exerted directly on the erythrocyte band 3 PTK or indirectly via another mechanism

TABLE IV

Effect of hydrogen peroxide and adriamycin on red blood cell glycolysis and band 3 peptide phosphorylation

Lactate production was measured in washed red blood cells after exposure to H_2O_2 and/or adriamycin as described under "Materials and Methods." Band 3 peptide phosphorylation was measured in resealed red blood cells containing $[\gamma\text{-}^{32}P]$ATP and band 3 peptide after exposure to H_2O_2 and/or adriamycin as described under "Materials and Methods."

	Lactate production	Peptide phosphorylation
	mM^a	cpm^b
Control	7.33	2,201
	7.62	2,342
H_2O_2 (1 mM)	14.39	21,020
	13.80	17,942
H_2O_2 (1 mM) + adriamycin (0.1 μM)	7.25	4,519
	6.32	3,697

[a] Concentration/liter of packed cells.
[b] Background levels determined in cells resealed in the absence of the band 3 peptide averaged 646 cpm.

such as a transmembrane redox system is presently not known. It is of interest to note that using a partially purified preparation of the band 3 PTK none of the oxidants directly stimulated the kinase.[4] In fact, as noted above, ferricyanide actually inhibits the kinase in cell-free systems. In addition, we have also observed that only hydrogen peroxide inhibits the band 3 PTPase in cell free assays, and in this case adriamycin fails to reverse the inhibition.[4] Thus, it appears that an intact cell system is necessary for the stimulation of band 3 tyrosine kinase activity by these mild oxidants as well as for the inhibition of this stimulation observed with adriamycin.

Finally, we wished to determine which steps in glycolysis were affected by the phosphorylation of band 3. The hypothesis that erythrocyte glycolysis could be regulated by tyrosine phosphorylation of an enzyme-binding site on band 3 predicted that phosphorylation-induced changes in substrate flow down the glycolytic pathway would be most pronounced at the step where enzyme binding to band 3 was most affected. G3PDH was clearly the logical candidate for this enzyme since (i) it was the only glycolytic enzyme to isolate with red cell ghosts in appreciable quantities (31, 32), (ii) it exhibited slightly greater affinity for ghosts and band 3 *in vitro* than the other possible enzyme ligands (14, 33, 34), and (iii) it was known to be at least ⅔ bound to the membrane in resting red cells *in vivo* (11, 35). To determine whether the G3PDH step was indeed being activated as a result of band 3 phosphorylation, the changes in the concentrations of various glycolytic intermediates were determined as a function of exposure to 0.4 mM $Fe(CN)_6^{-3}$. As shown in Fig. 4, all intermediates preceding the G3PDH step in glycolysis were seen to decrease upon ferricyanide stimulation, while metabolites following the G3PDH step were observed to increase in concentration. This suggests that the $Fe(CN)_6^{-3}$ control of glycolysis does in fact occur at the G3PDH step, as has been previously suggested (24). Whereas normal feedback control of erythrocyte glycolysis is undoubtedly centered at the classic phosphofructokinase and hexokinase steps (36–38), tyrosine kinase regulation of glycolysis appears to operate predominantly through the release and consequent activation of G3PDH. Whether other mild oxidants which activate glycolysis (39) operate by a similar mechanism is unknown.

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[4] M. Harrison, C. Isaacson, R. Geahleu, and P. Low, unpublished observations.
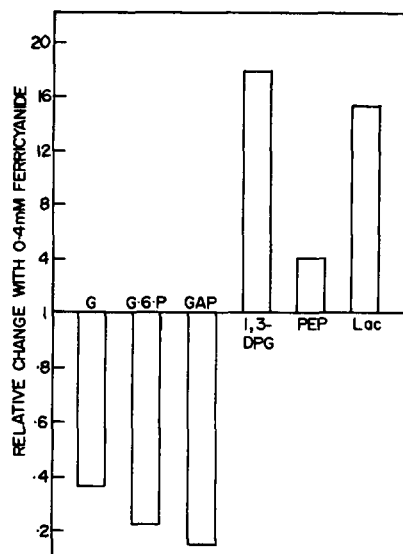
FIG. 4. **Relative change in the concentration of various glycolytic intermediates upon stimulation of erythrocytes with Fe(CN)$_6^{-3}$.** Intact red cells were incubated for 10 min at 37 °C in Ringer's buffer supplemented with 0.4 mM $K_3Fe(CN)_6$ prior to quenching with 1 M $HClO_4$ and analysis of glycolytic intermediates (15). The site of glycolytic regulation by Fe(CN)$_6^{-3}$ is identified as the step where the transition from substrate depletion to substrate accumulation occurs, *i.e.* the cross-over point. For Fe(CN)$_6^{-3}$ this occurs at the G3PDH step. The acronyms represent *G*, glucose; *G6P*, glucose-6-phosphate; *GAP*, glyceraldehyde-3-phosphate; *1,3-DPG*, 1,3-diphosphoglycerate; *PEP*, phosphoenolpyruvate; *Lac*, lactate.

DISCUSSION

We have presented evidence that agents that promote the tyrosine phosphorylation of the G3PDH-binding site on band 3 stimulate erythrocyte glycolysis. It is unlikely that these oxidizing agents directly activate G3PDH since the enzyme has a highly reactive catalytic sulfhydryl and reagents which oxidize or modify this sulfhydryl inactivate the enzyme (40, 41). The qualitative correlation between native band 3 tyrosine phosphorylation (Fig. 1) and elevation of red cell glycolysis suggests that some relationship between the phosphorylation state of band 3 and glycolysis must exist. This conclusion is further strengthened by the more quantitative data using the band 3 peptide assay (Fig. 3) as well as the simultaneous reversal of both effects by adriamycin. Studies of Tsai *et al.* (14) showing the quantitative inhibition of G3PDH upon band 3 binding (14) and our work demonstrating significant blockade of G3PDH binding upon band 3 tyrosine phosphorylation at the G3PDH-binding site (6) suggest the likely molecular basis of this correlation, *i.e.* tyrosine phosphorylation of the G3PDH site on band 3 activates G3PDH by blocking its inhibitory interaction with the membrane.

We have proposed a mechanism whereby the tyrosine phosphorylation of band 3 could contribute to the regulation of glycolysis in red blood cells. The actual physiological conditions under which this system operates have yet to be demonstrated. Because of difficulties in accurately and reproducibly quantifying the extent of band 3 tyrosine phosphorylation in our system, we have not determined the stoichiometry of band 3 phosphorylation and do not know the fraction of native band 3 that is phosphorylated in response to the various stimulants. Still, if the mechanism we propose is valid, then enough band 3 must be phosphorylated to activate G3PDH. Human erythrocytes contain significantly fewer tetramers of G3PDH (3×10^5 tetramers/cell) than monomers of band 3 (1.2×10^6/cell) (35). It is unlikely, however, that much of the

residual band 3 capacity is available to G3PDH, since the N-terminal sequence of band 3 is also known to bind aldolase, phosphofructokinase, hemoglobin, and ankyrin (6, 12, 42). Because of these multiple interactions, it is difficult to predict the extent to which band 3 must be phosphorylated to achieve the 2-fold stimulation of glycolysis observed in these studies. If most of the G3PDH were membrane bound (11, 35) and if the band 3 PTK specifically associated with the subpopulation of band 3 that binds G3PDH, then a relatively small fraction of band 3 would have to be phosphorylated to promote the observed doubling of glycolytic rates. It has been suggested that the band 3 kinase associates with only the subset of band 3 molecules that preferentially bind to the red cell skeleton (17). G3PDH has also been reported to associate with this same subset of band 3 molecules, under certain conditions (17 and references herein). Thus, we think it likely that in response to oxidants at least G3PDH is being activated by its displacement from band 3. Whether other mechanisms also contribute to the observed stimulation of glycolysis is presently unknown. Experiments are currently underway to test these various possibilities.

The effect of hydrogen peroxide on band 3 tyrosine phosphorylation is of interest because this reagent has long been known to mimic insulin action (43, 44). Since the insulin receptor is a PTK, many of insulin's effects are thought to be mediated through tyrosine phosphorylation (for a review see Ref. 45). Two recent reports have shown that hydrogen peroxide increases the phosphotyrosine content of several putative cellular substrates of the insulin receptor kinase (22, 46), possibly by inhibiting a PTPase (22). Our results are certainly consistent with hydrogen peroxide acting through the inhibition of a band 3 PTPase. It is of interest to note that red cell membranes have been shown to produce hydrogen peroxide in the presence of NADH and O_2 (47).

The fact that 0.1 μM adriamycin was found to quantitatively return both band 3 tyrosine phosphorylation and lactate production to basal levels raises a possible question regarding its site and mechanism of action in cancer therapy. Although most workers have focused on adriamycin's ability to distort DNA and perturb gene expression (48–50), mature erythrocytes have neither DNA nor the capability to process genetic information. The fact that adriamycin prevented an elevation of band 3 tyrosine phosphorylation coupled with its reported ability to inhibit PTKs may be significant in its therapeutic role in cancer treatment. In view of the observation that adriamycin-linked dextran beads retain their cytotoxicity to tumor cells (51), the possibility of a membrane site of adriamycin action perhaps deserves renewed consideration.

Finally, it is becoming increasingly clear that band 3 is not an abundant protein in nonerythroid cells (52–54). However, most metabolic enzymes which were once thought to be entirely soluble in a cell's cytoplasm are now believed to be at least partially associated with structures such as microtubules, microfilaments, and cytoplasmic domains of integral membrane proteins (55–57). In view of this similarity, it is interesting to note that activation of growth factor receptors, which is commonly accompanied by an elevation of tyrosine phosphorylation, usually also leads to elevation of metabolism (58). Furthermore, in a recent study of human mammary tissue from normal individuals and patients with benign and malignant tumors, a linear relationship extending over two orders of magnitude was observed between tyrosine kinase activity and soluble aldolase activity (59). Whether these observations derive from an enzyme release/activation mechanism related to that in red cells is not clear, however, the possibility in our minds merits some consideration.

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