Identification of Fructosamine Residues Deglycated by Fructosamine-3-kinase in Human Hemoglobin*

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Fructosamine-3-kinase (FN3K) phosphorylates fructosamine residues, leading to their destabilization and their shedding from protein. Support for the occurrence of this deglycation mechanism in intact cells has been obtained by showing that hemoglobin is significantly more glycated when human erythrocytes are incubated with an elevated glucose concentration in the presence of 1-deoxy-1-morpholinofructose (DMF), a cell-permeable inhibitor of FN3K, than in its absence. The aim of this work was to identify the fructosamine residues on hemoglobin that are removed as a result of the action of FN3K in intact erythrocytes. Highly glycated hemoglobin derived from intact human erythrocytes incubated for 48 h with 200 mM glucose and DMF was incubated in vitro with FN3K and [γ-32P]ATP. After reduction of fructosamine 3-phosphates with borohydride, the protein was digested with trypsin. Peptides were separated by reversed-phase high-performance liquid chromatography, and the radioactive peaks were analyzed by mass spectrometry. Nine different modified residues were identified. These were Lys-α-16, Lys-α-61, Lys-α-139, Val-β-1, Lys-β-17, Lys-β-59, Lys-β-66, Lys-β-132, and Lys-β-144. Some (e.g. Lys-α-139) were readily phosphorylated to a maximal extent by FN3K in vitro whereas others (e.g. Val-β-1) were slowly and only very partially phosphorylated. The radiolabeled peptides containing reduced fructosamine 3-phosphates bound to Lys-α-16, Lys-α-139, and Lys-β-17 were much less abundant if the hemoglobin substrate used for the in vitro phosphorylation with FN3K and [γ-32P]ATP came from erythrocytes incubated with an elevated glucose concentration in the absence of DMF, indicating that these lysine residues had been substantially deglycated in intact cells when FN3K action was unrestrained. Other residues (e.g. Val-β-1, Lys-α-61) seemed to be insignificantly deglycated in intact cells.

It has long been assumed that proteins, being easily renewed, are not repaired, unlike DNA, where mutations can threaten the life of the cell. On the contrary, it has become clear that some spontaneous or chemically induced modifications of proteins can be remedied by specific enzymatic systems. Protein-methionine-S-oxide reductase reverses oxidative damage caused to methionine residues (1), whereas protein-L-isoaaspartate O-methyltransferase permits the conversion of L-isoaaspartate residues, which mostly result from a spontaneous rearrangement of asparagines, to L-aspartate residues (2). Disruption of the gene for either of these enzymes in mice results in a diseased state and in a decreased lifespan (3, 4), indicating the physiological importance of these protein repair mechanisms.

One of the chemical alterations that proteins undergo is glycation, which occurs from sugars. Glucose and other free aldoses spontaneously react with N-terminal and ε-amino groups of proteins to form Schiff bases, which slowly rearrange to ketosamines or, if the reacting sugar was glucose, fructosamines. The spontaneous fate of fructosamines is to further react slowly and become ‘advanced glycation end products,’ which are thought to play a role in the pathophysiology of several disorders in man, most particularly diabetes (5–7).

Recent evidence indicates that fructosamines, or at least some intracellular protein-bound fructosamines, can be repaired by a newly identified enzyme, fructosamine-3-kinase (FN3K) (3, 9). The latter phosphorylates both free and protein-bound fructosamines on their third carbon, making them unstable (half-life of about 5–7 h; Ref. 9) and causing them to detach from proteins as 3-deoxyglucosone, with concomitant formation of inorganic phosphate and regeneration of the amine in its pristine state. Human erythrocytes, which have a long lifespan (about 120 days in man) and no protein synthesis, are particularly rich in FN3K and thus represent a convenient model to study protein deglycation in ex vivo experiments. Using a cell-permeable competitive inhibitor of FN3K, 1-deoxy-1-morpholinofructose (DMF), we indeed confirmed that FN3K phosphorylates glycated hemoglobin in intact cells and that this leads to its partial deglycation (10). However, the identity of the fructosamine residues in hemoglobin that underwent deglycation was not determined. This information is important because it could indicate the types of fructosamine residues that can be removed from proteins and those that cannot, thereby helping in the understanding of the physiological significance of deglycation. Furthermore, it could provide an explanation for the long-standing observation that some residues...
of hemoglobin (e.g. Lys-a-16) that are particularly well glycated when the pure protein is incubated with glucose are poorly glycated in vitro (11).

The aim of this work was to identify the fructosamine residues that are removed from hemoglobin in intact erythrocytes as a result of the action of FN3K. Such residues should be absent from hemoglobin derived from erythrocytes incubated with high glucose in the absence of DMF but should be present when erythrocytes are incubated with high glucose in the presence of DMF. When present on hemoglobin, these residues should be readily phosphorylated by FN3K in vitro, which would allow their tagging with radiolabeled ATP and thereby facilitate their identification.

**EXPERIMENTAL PROCEDURES**

**Synthesis of Fructosamines and FN3K Activity Measurement—** Fructose-6-(1-deoxyfructosylaminol) and fructose-6-(1-deoxyfructosylglycyl) (N-ε-(1-deoxyfructosylglycyl)glycine) were synthesized according to Mossine et al. (12). In brief, a mixture containing 20 mmol of anhydrous p-glucose in 6 ml of methanol and 4.5 ml of glycerol was refluxed during 30 min before the addition of 7.5 mmol of the corresponding amino acid, after which the reflux was continued for about 20 h (fructose-6-valine) or 8 h (fructose-6-glycylglycine). The sample was then diluted with water to a final volume of 50 ml and chromatographed on a 10-ml cation-exchange column (Amberlite, H⁺ form). After extensive washing with water, the Amadori compound was eluted with 0.25 M NH₄OH. Eluting fractions positive to both ninhydrin and reducing sugars (13) were pooled, concentrated under vacuum, decolorized with charcoal, and further purified by gel filtration on a Biogel P2 column (0.9 × 60 cm) equilibrated with water. About 3 mmol (as estimated by assaying reducing sugars) of purified products was thereby obtained. Structures were checked by electrospray tandem mass spectrometry (electrospray ionization-MS/MS). FN3K activity toward these two fructosamines was assayed as described for fructosylglycine (N-ε-(1-deoxyfructosylglycyl)glycine) (8).

**Ex Vivo Incubation of Intact Human Erythrocytes Suspensions and Partial Purification of Hemoglobin—** Incubations of human erythrocytes were carried out as described previously (10). In brief, suspensions (1/20 in a Krebs-Henseleit buffer) were incubated at 37 °C for 48 h in the presence of 5 or 200 mM glucose, with or without 5 mM DMF. Incubations were stopped by centrifugation, and the red blood cells were washed and then kept at −20 °C before use. Hemoglobin was partially purified from these erythrocyte lysates by anion-exchange chromatography on DEAE-Sepharose (10).

**In Vitro Phosphorylation of Hemoglobin by FN3K—** About 0.35 mg of partially purified hemoglobin was incubated at 30 °C with 0.05 μM of purified recombinant human FN3K (8) in 0.25 ml of a medium containing 20 mM Tris/HCl, pH 7.8, 1 mM dithiothreitol, 1 mM EGTA, 0.2 mM ATP-Mg, and 10⁶ cpm (experiment shown in Fig. 1) or 30 × 10⁶ cpm (other experiments) of [γ⁻³²P]ATP (Amersham Biosciences). For the experiment shown in Fig. 1, 25-μl aliquots of the medium were spotted at the indicated times on cation-exchange papers (P81; Whatman), which were washed three times with ice-cold 75 mM H₃PO₄ and then once with alcohol and once with acetone. After drying, the papers were counted for radioactivity in the presence of scintillant. For the other experiments, the incubation was stopped by the addition of 0.5 μM Tris, pH 7.8, and 1 mM NaBH₄ to give final concentrations of 50 and 100 mM, respectively. Incubation was continued at room temperature for 20 min, then on ice for 40 min. Proteins were precipitated with one vol of 10% trichloroacetic acid and twice with acetone (on ice). The protein pellet was washed twice with 10% trichloroacetic acid and twice with acetone (on dry ice), before drying under vacuum and resuspension in 75 μl of 0.1 M urea, and 100 μM Tris, pH 8. Trypsin (sequencing grade, from Promega; 1 μg/500 μg of hemoglobin) was then added and incubation was performed overnight at 30 °C.

**Separation of Tryptic Peptides by HPLC and Mass Spectrometric Analysis—** Peptides were separated by reverse phase narrowbore HPLC on a Vydac C18 column (2.1 mm × 25 cm) with an acetonitrile gradient in 0.1% (v/v) trifluoroacetic acid (solvent A). Elution was performed with the following gradient program: 5–100% solvent B (70% (v/v) acetonitrile in solvent A) over 100 min at a flow rate of 200 μl/min generated by a model 1100 Agilent solvent delivery system. Rechromatography peaks were detected by Cerenkov counting (Fig. 2) or liquid scintillation counting (Fig. 4), dried down under vacuum, redissolved in 5 μl of 50% (v/v) acetonitrile/0.3% (v/v) acetic acid and analyzed by nano-electrospray ionization tandem mass spectrometry (nano-electrospray ioniza-

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**RESULTS**

**In Vitro Phosphorylation of Glycated Hemoglobin with FN3K—** It was previously shown that the presence of DMF, an inhibitor of FN3K, increased the accumulation of glycated hemoglobin from 13 to 20% in 48-h incubations of intact human erythrocytes in the presence of 200 mM glucose. No such effect was observed in incubations carried out with 5 mM DMF. The effect of DMF observed in the presence of 200 mM glucose has been attributed to a decrease in deglycation as a result of inhibition (by DMF) of FN3K-mediated phosphorylation of fructosamine residues. Therefore, hemoglobin from erythrocytes incubated in the presence of 200 mM glucose with 5 mM DMF was more phosphorylated in vitro by FN3K than hemoglobin derived from erythrocytes incubated with the same concentration of glucose but in the absence of DMF (10).

Fig. 1 confirms and extends these findings. A maximal incorporation of about 0.12 mol of phosphate per mol of hemoglobin tetramer was achieved for hemoglobin from cells incubated with 200 mM glucose and 5 mM DMF, compared with about 0.07 for hemoglobin from cells incubated with the same glucose concentration but without DMF. Lower but significant degrees of phosphorylation were observed with hemoglobin from erythrocytes incubated with the same concentration of glucose but in the absence of DMF (10).

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**FIG. 1. Time course of human hemoglobin phosphorylation by human recombinant FN3K: effect of incubation of erythrocytes with a high glucose concentration with or without DMF.** Hemoglobin from erythrocytes incubated for 48 h with 5 or 200 mM glucose in the absence or in the presence of 5 mM DMF was incubated at 30 °C in the presence of FN3K and [γ⁻³²P]ATP as described under “Experimental Procedures.” At the indicated times, aliquots were spotted on phosphocellulose papers for measurement of ³²P-incorporation. The results are the means ± S.E. of four experiments.
Identification of the Phosphorylated Fructosamines—To perform this identification, the four samples of hemoglobin described above were incubated with \([\gamma\text{-}^{32}\text{P}]\text{ATP}\) and FN3K for 180 min at 30 °C. Under these conditions, only a small proportion (<15% if a half-life of 12 h at 30 °C is assumed) of the fructosamine 3-phosphates formed is expected to be degraded. The samples were then reduced with sodium borohydride to stabilize the ketosamine 3-phosphates by converting them to hebitolamine 3-phosphates and were digested by overnight incubation with trypsin. Radioactive peptides were separated by reverse-phase HPLC and counted to quantify the radioactivity associated with each of them and to facilitate their identification by mass spectrometry. As shown in Fig. 2, at least 10 different peaks of radioactivity could be resolved, indicating that glycated hemoglobin is phosphorylated by FN3K on multiple residues. It is interesting that comparison of the profiles indicated that the presence of DMF during incubation of erythrocytes in the presence of 200 mM glucose affected some of the peaks (mainly peaks V, VII, X, and XII) but not others (peaks I, IV, VI, IX; central part of peak VIII), suggesting that during the incubation of erythrocytes in the absence of DMF, certain residues had been significantly phosphorylated by FN3K and consequently deglycated. The profiles corresponding to incubations with 5 mM glucose with or without DMF were similar, confirming that DMF had no detectable effect at the low glucose, with or without 5 mM DMF (closed versus open symbols, respectively). Hemoglobin was then partially purified on DEAE-Sepharose and incubated with FN3K and \([\gamma\text{-}^{32}\text{P}]\text{ATP}\) for 3 h in vitro. After reduction of fructosamine 3-phosphate residues with borohydride, protein was precipitated, resuspended in urea, and digested overnight with trypsin. Peptides were separated by narrowbore reversed-phase HPLC in an acetonitrile gradient. Fractions corresponding to radioactive peaks eluting from the column (I to XII) were analyzed by mass spectrometry (see Table I).

To facilitate the identification of the peptides present in these peaks by mass spectrometry, a list of their expected m/z ratios was calculated by assuming 1) an increase of 244 Da in the mass of an amino acid residue modified by a reduced fructosamine 3-phosphate group; 2) that conversion of a lysyl residue to a hebitolysyl 3-phosphate residue would prevent cleavage by trypsin, as is known for hebitolysyl residues (11, 14). Cleavage would then occur at a neighboring lysyl (or arginyl) residue, because the probability of having two glycated lysyl residues next to each other in the same polypeptide chain is low. Full MS spectra recorded for each of the peak fractions were inspected for the presence of each of the potential peptides listed in Table I, either as singly, doubly, or triply charged ions. Each candidate ion identified in this way was analyzed by tandem mass spectrometry (MS/MS). Phosphorylated peptides were identified by neutral loss of 98 Da, corresponding to the removal of phosphoric acid (H₃PO₄) by β-elimination and resulting in a peptide that was dehydrated at the level of the phosphorylated residue. Confirmation of the sequence of each peptide was obtained by comparing the m/z values of its fragments obtained in MS/MS mode and/or the m/z values of fragments obtained from the ‘dephosphorylated peptide’ in MS³ mode (see Fig. 3 as an example). This information was also used to verify that the phosphorylated residue corresponded to a reduced fructosamine (hebitolysine in most cases; hebitolvaline for the N-terminal peptide of the β-chain) and not to any other potential phosphorylated residue (serine, threonine, etc.).

Using this strategy, we identified a phosphorylated peptide and the corresponding phosphorylated fructosamine in six of the peaks shown in Fig. 2 (Table I). In peak VIII, two different peptides were identified in the peak fractions (fractions 37–39), in which the modified residues were Lys-β-59 and Lys-β-66, respectively. An analysis of the descending limb of peak VIII (fraction 40), which showed a different sensitivity to DMF than the peak fraction (see Fig. 2), enabled us to identify Lys-β-17. The peak fractions in which no peptide could be identified by nano-electrospray ionization-MS were analyzed by matrix assisted laser desorption ionization-Quadrupole-time of flight MS, leading to the identification of the phosphorylated residue in peak XI.

In all, we were able to identify the phosphorylated fructosamines in almost all significant peaks (except peak VI) in the first part of the chromatogram, where short peptides are expected to elute. Only two of the peaks eluting after fraction number 40 could be identified. DMF-sensitive peaks corresponded to peptides containing the following glycated residues: Lys-α-16, Lys-α-139, and Lys-β-17 (peak V, VII, and descending limb of peak VIII, respectively). The DMF-insensitive peaks corresponded to Val-β-1, Lys-β-144, Lys-α-61 (peak I, IV, and IX, respectively). The two main peptides present in peak VIII most likely correspond to two sites (Lys-β-59 and Lys-β-66). The status of peak XI, containing Lys-β-132 is uncertain, because of potential overlapping with other peaks.

Time Course of in Vitro Phosphorylation of the Glycated Resides—In an attempt to determine the relative affinity of FN3K for the various fructosamine residues in hemoglobin, we studied the time course of their in vitro phosphorylation by this enzyme. The best substrates would be expected to be maximally phosphorylated at shorter times than lower affinity substrates. Hemoglobin from erythrocytes that had been incubated with DMF and 200 mM glucose was treated for various times with \([\gamma\text{-}^{32}\text{P}]\text{ATP}\) and FN3K, reduced with borohydride, digested with trypsin, and analyzed by HPLC (Fig. 4). The time course of phosphorylation of the various peaks was distinct, some being almost maximally phosphorylated after 15 min (e.g. peak IV, VII, and X), whereas other peaks were almost linearly phosphorylated during the whole incubation period (e.g. peaks I, V, VI, IX). Some of these data for relatively well-defined peaks are shown as a time course in Fig. 5. Residues Lys-17,
TABLE I  
Sequence and monoisotopic mass of phosphopeptides expected from digestion with trypsin of glycated hemoglobin phosphorylated by FN3K

| Modified residue | Sequence of the corresponding phosphopeptide | Calculated monoisotopic mass for MH+ | Identified in peak no. (see Fig. 2) |
|------------------|---------------------------------------------|-------------------------------------|-----------------------------------|
| α-chain          |                                             |                                     |                                   |
| Val-1            | VLPADK                                      | 973.45                              | Da                                 |
| Lys-7            | VLPADKTVNK                                  | 1415.71                             |                                    |
| Lys-11           | TVKAAAGK                                    | 1218.58                             |                                    |
| Lys-16           | AAQKVQDVRAGEYGAELER                        | 2287.04                             | V                                  |
| Lys-40           | MF6KPTKTTYPPHDFLSHSGAQVK                   | 3130.47                             |                                    |
| Lys-56           | TYPFHPDLSHSGAQVKGHGK                      | 2457.13                             |                                    |
| Lys-60           | GRHGK                                       | 770.35                              |                                    |
| Lys-61           | KVADALTNAVHVDMPNALSALSDLHAKR              | 3668.62                             | IX                                 |
| Lys-90           | VADALTNAVHVDMPNALSALSDLHAKR              | 3569.71                             |                                    |
| Lys-99           | VDPNVNLKSICLCLVTLLAAHLPAEFPPAPVHSADL      | 4011.07                             |                                    |
| Lys-127          | LSHCICLVTLLAAHLPAEFPPVHASL2K               | 4445.35                             |                                    |
| Lys-139          | FLASVSTVLTKYR                               | 1815.92                             | VII                                |
| β-chain          |                                             |                                     |                                    |
| Val-1            | VHLTPEEK                                    | 1196.55                             | I                                  |
| Lys-8            | VHLTPEEKSATVALWKG                           | 2110.05                             |                                    |
| Lys-17           | SAVTALNGKVNVDEVGQALGR                      | 2472.21                             | VIII*                              |
| Lys-59           | FFESFGDLSTPDAVGMNPVVK                      | 2530.15                             | VIII                               |
| Lys-61           | VKAHGK                                      | 883.43                              |                                    |
| Lys-65           | ANQKG                                       | 784.36                              |                                    |
| Lys-66           | KVLAGFSDGLDHDLNK                           | 2042.02                             | VIII                               |
| Lys-82           | VLGAFSGDLHDLNLKGTATPSLSDKC                 | 3316.58                             |                                    |
| Lys-95           | GTPATPSLSDKCXLHVDOPFR                      | 2773.26                             |                                    |
| Lys-120          | LLGHVLCVLLAMHPQDEYFPPPQAYYQ                | 3223.69                             |                                    |
| Lys-122          | EFTPPQYQAYQKOVAVGNALAKY                    | 2753.4                              | XI                                 |
| Lys-144          | VVAVGAVNALAIYK                             | 1693.84                             | IV                                 |

* Lys-β-17 was identified on the right edge of peak VIII, in the second fraction after the peak fraction.

Lys-59, and Lys-66 of the β-chain have been omitted from this figure because they were identified in the same radioactively labeled peak (VIII) and therefore cannot be discriminated with respect to their phosphorylation rate. The same is true for Lys-β-132, for which the corresponding peptide probably overlaps with unidentified phosphopeptides.

Dose Dependence of in Vitro Phosphorylation of Hemoglobin by FN3K—The differences in the sizes of the peaks noted when comparing the profiles shown in Fig. 2 most probably resulted from differences in the degree of glycation at each of the possible sites in hemoglobin. To verify that, for each of the peaks, the degree of phosphorylation correlated with the glycation extent, we phosphorylated in parallel 1) hemoglobin derived from erythrocytes incubated with 5 mM glucose, 2) hemoglobin derived from differences in the degree of glycation at each of the positions of interest, and 3) a composite sample containing 50% of both. These samples, which contained identical amounts of hemoglobin, were digested with trypsin to examine the HPLC labeling profile. The profile corresponding to the composite sample was intermediate between the other two and almost corresponded to their arithmetic mean, indicating that for each of the peptides, phosphorylation was proportional to the amount of fructosamine (not shown).

Affinity of FN3K for Low Molecular Weight Substrates—Glycated Val-β-1 was only slowly phosphorylated by FN3K (see Fig. 5). We therefore decided to compare the affinity of FN3K for the corresponding low-molecular-weight Amadori compound, fructosevaline (N-α-(1-deoxyfructosyl)-valine), and for a glycated dipeptide, fructoseglycylglycine (N-α-(1-deoxyfructosyl)-glycylglycine) with its affinity for free fructoselysine (Nε-(1-deoxyfructosyl)-lysine). FN3K displayed a lower affinity for the first two compounds (Km = 0.8 and 1.5 μM, with Vmax of 14.8 and 14.5 nmol/min/mg, respectively) than previously determined for fructoselysine (Km of 7.2 μM, and Vmax of 18 nmol/min/mg (8)).

DISCUSSION

Identification of Glycated and Phosphorylated Amino Acid Residues in Hemoglobin by Mass Spectrometry—This article presents the first mass-spectrometric identification of glycated residues in human hemoglobin that are phosphorylated by FN3K. This identification is based on a mass increment that corresponds to a reduced (by treatment with borohydride) fructosamine phosphate, the loss of phosphoric acid at low energy collision-induced dissociation, and fragmentation at high energy collision-induced dissociation to localize the fructosamine phosphate residue. Nine different fructosamine residues phosphorylated by FN3K were identified in this way. It is interesting that most of these residues correspond to glycation sites previously identified (Table II) on in vitro or in vivo glycated hemoglobin (11, 15). Lys-α-16, Lys-α-61, Val-β-1, Lys-β-17, and Lys-β-66 were previously unambiguously assigned, but for Lys-α-139, Lys-β-132, and Lys-β-144, the allocation was tentative. We identified Lys-β-59 as a novel glycation site. The following previously established glycation sites Val-α-1, Lys-α-7, Lys-α-40, and Lys-β-120 were not detected presumably because of 1) low level of glycation (Val-α-1, Lys-α-7, Lys-α-40, Lys-β-120), 2) long length of the modified peptide (Lys-β-120), and 3) low affinity for FN3K (Val-α-1, see below). It must be emphasized that no present technique can provide an exhaustive identification of glycation sites in a protein, mainly because of low stoichiometry of glycation and its distribution among a number of different residues. In the present study, the total stoichiometry of phosphorylation was about 0.12 mol/mol of tetramer, which means that if a dozen different peptides were labeled, the mean individual phosphorylation stoichiometry was about 1% per residue.

Difference in the Affinity of FN3K for Fructosamine Residues in in Vitro Incubations—The rate of in vitro phosphorylation of the various glycated residues in hemoglobin differs substantially (see Fig. 5). Some of the residues seem to be rapidly phosphorylated to a maximal extent (Lys-β-144, Lys-α-139),
FIG. 3. Identification of glycated and phosphorylated Lys-α-139 in hemoglobin by nano-electrospray ionization-MS/MS. A, MS² spectrum of a doubly charged phosphopeptide ion (m/z 908.5), expected in the case of Lys-α-139 modification by a reduced fructosamine 3-phosphate group (see Table I). A loss of 98 Da is observed, and the y₃ and y₂ fragments have a mass difference of 372.15 Da corresponding to the mass of a hexitollysyl 3-phosphate residue, thus confirming modification of Lys-α-139.

B, MS³ spectrum of the ion arising from loss of 98 Da is shown (m/z 859.54 of the doubly charged ion in A). The y₃ and y₂ fragments have a mass difference of 274.0 Da (see inset in B) that corresponds to anhydrohexitollysine. KHXN-P denotes the hexitollysyl 3-phosphate residue in the peptide sequence.
Fructoseglycine, fructoseglycylglycine, and fructosevaline,
Lys-

purified hemoglobin from human erythro-
in vitro
susible in the hemoglobin tetramer (Fig. 6

course of

However, hemoglo-
in vitro

is surprising, because this residue seems to be inacces-

extent of glycation. The difference may be related to the acces-

although in this case, we are unable to have a good estimate of

could also explain why Val-

prevents binding of fructosamines by steric hindrance. This

phosphorylation extent is only about 3%, which suggests that it is at

least 30-fold poorer as a substrate compared with Lys-

phosphorylation by FN3K during the 48-h incubation in the absence

cose and DMF than from erythrocytes incubated with high

cated as a consequence of their phos-

sites from peaks eluting late in the chromatogram. It is inter-

and Lys-

chains. Their location at a

in vitro

were stopped and the samples

then processed as described for the experiment shown in Fig. 2.

was not identified in the

present study), suggesting that the

alpha-carbonyl group prevents binding of fructosamines by steric hindrance. This

could also explain why Val-

was not identified in the

present study. Moreover, the amino-terminal valine of the

chain extremity could facilitate their access to the catalytic site

of FN3K. The disadvantage of this approach is that the amount of

activity found in different peaks in the same chromatogram
do not reflect the relative glycation extent at the corres-

corresponding residues, because of differences in the affinity

FN3K for the various glycated residues. However, comparison

of the same peak of radioactivity in different chromatograms is

legitimate, allowing one to compare the degree of glycation of the

residue from one sample to another. This is

by the mixing experiment, which indicates that, for

each of the peaks, the incorporated radioactivity is proportional to the

concentration of glycated residue.

The observation that some of the phosphorylated peptides

are severalfold more abundant in the phosphorylation product

of hemoglobin from erythrocytes incubated with 200 mM glu-
cose and DMF than from erythrocytes incubated with high

glucose in the absence of inhibitor indicates that the corre-

cessing that Lys-

was previously shown to be a major gly-

neine and fructoseglycylglycine, such as

fructose-1,6-diphosphate (Fig. 6, A and B) (17, 18), which might therefore

intracellular concentration). Lys-

-139, is close to the binding site for 2,3-bisphos-

phosphorylation by FN3K under in vitro conditions, although in this case, we are unable to have a good estimate of

this difference because of the lack of information on their extent of glycation. The difference may be related to the acces-
sibility of the residue and the flexibility of the peptide chain in

which it is located. It is noteworthy in this respect that the two

most rapidly phosphorylated glycated lysines are the antepe-

nultimate residues of the 

- and 

chains. Their location at a

chain extremity could facilitate their access to the catalytic site

of FN3K.

The finding that glycated Lys-

-132 can be phosphorylated

in vitro

is surprising, because this residue seems to be inacces-
sible in the hemoglobin tetramer (Fig. 6A). However, hemoglo-

bin readily dissociates into dimers (16), particularly in dilute

solutions (the concentration of hemoglobin was about 1.5 mg/ml

in the cell-free incubation, which represents about 1/200 of the

Partially

phenolic residues present in radioactive peaks shown in Fig. 4.

Values shown in this figure correspond to peak fractions of the profile

shown in Fig. 4. See text for details.

whereas others are phosphorylated at a much lower rate and

presumably incompletely. This would be especially true for

Val-

. Assuming that this residue represents about 30% of

the total glycation sites (15), it can be calculated that its phos-

phorylation extent is only about 3%, which suggests that it is at

least 30-fold poorer as a substrate compared with Lys-

or Lys-

-139.

This is in agreement with the fact that FN3K has a

much lower affinity (about 100-fold) for low-molecular-

weight fructosamines bound to an 

-amino group, such as

fructoseosamine, fructoseglycylglycine, and fructoseosamine,

than to an 

-amino group or to morpholine (8, 9; results from

the present study), suggesting that the 

-carbonyl group prevents binding of fructosamines by steric hindrance. This

could also explain why Val-

was not identified in the

present study. Moreover, the amino-terminal valine of the

chain is about 10-fold less glycated than the same residue in the

chain (15) (Table II).

There is also a difference among glycated lysines of hemoglo-

bin in their affinity for FN3K under in vitro conditions, although in this case, we are unable to have a good estimate of

this difference because of the lack of information on their extent of glycation. The difference may be related to the acces-
sibility of the residue and the flexibility of the peptide chain in

which it is located. It is noteworthy in this respect that the two

most rapidly phosphorylated glycated lysines are the antepe-

nultimate residues of the 

- and 

chains. Their location at a

chain extremity could facilitate their access to the catalytic site

of FN3K.
TABLE II
Comparison of the sites of glycation of human hemoglobin identified in the present study with those previously described

| The present study | Shapiro et al., 1980 | Hemoglobin glycated in vitro | Hemoglobin glycated in vivo | Zhang et al., 2001 | Hemoglobin glycated in vivo | Relative abundance |
|------------------|---------------------|-----------------------------|----------------------------|---------------------|-----------------------------|-------------------|
| α-Chain          |                     |                             |                             |                     |                             |                   |
| Lys-16           | Val-1 (5)           | Val-1 (5)                   |                             | Val-1              |                             | 0.01              |
|                  | Lys-6 (6)           |                             |                             | Lys-7 or Lys-16    |                             | 0.01              |
|                  | Lys-16 (2)          |                             |                             | Lys-7 or Lys-16    |                             | 0.01              |
| Lys-61           | Val-1 (1)           | Lys-17 (4)                  |                             | Val-1              |                             | 0.32              |
| Lys-59           | Lys-66 (3)          | Lys-66 (2)                  |                             | Lys-8 or Lys-17    |                             | 0.01              |
| Lys-132          | Lys-144             |                             |                             | Lys-61 or Lys-65 or Lys-66 |                             | 0.09              |
| Lys-144          |                     |                             |                             | Lys-132 or Lys-144 |                             | 0.06              |
| β-Chain          |                     |                             |                             | Lys-132 or Lys-144 |                             | 0.06              |

Discussion

In the present work, glycation of a given residue is inferred from the fact that it is phosphorylated by FN3K in vitro (see Fig. 2 and Table I). Underlined residues in the first column are those for which we provide evidence for deglycation in intact erythrocytes (see “Discussion”). Identification of the sites of in vitro glycation of hemoglobin by Shapiro et al. (11) was by incubation of the unglycated protein with [3H]glucose, reduction of the fructosamine residues with borohydride, and amino acid analysis of the labeled peptides obtained after digestion with trypsin. The same approach was used for sites of in vivo glycation, except that the fact that in this case, glycated hemoglobin from a diabetic patient was first separated from the non-glycated protein by affinity chromatography, and [3H]borohydride was used in the reduction step. For both approaches, the order of prevalence regarding the extent of glycation among residues is indicated in parentheses. Zhang et al. (15) identified sites of in vivo glycation by LC-ESI-MS, in this case using glycated hemoglobin from a diabetic patient purified by affinity chromatography and digested with endoproteinases Glu-C and Asp-N. The relative abundance of the various glycated sites, as calculated from their data, is also shown.

Other glycated residues, such as Val-β-1 and Lys-α-61, do not seem to be efficiently deglycated in intact erythrocytes, despite the fact that they are phosphorylated in vitro by FN3K at a concentration that corresponds to about 30% of the physiological intracellular concentration of this enzyme. Although we cannot exclude the idea that, because of their microenvironment, some fructosamine 3-phosphates may lose their phosphate without being deglycated, other explanations can be proposed. The poor ability of Val-β-1 to be deglycated is largely the consequence of it being bound to an α-amino group, which, as mentioned above, makes it a poor substrate for FN3K. Steric hindrance by 2,3-bisphosphoglycerate could also prevent accessibility to FN3K. Why Lys-α-61 is a poor substrate could be that it is situated in helix E, which forms strong hydrophobic and van der Waals contacts with the heme prosthetic group, and would probably be inflexible. This could also apply to Lys-β-66, which was previously reported to be one of the main in vivo glycation sites of hemoglobin (11). Unfortunately, because it is present in the same peak as Lys-β-59, we are unable to decrease the accessibility of this residue in intact erythrocytes.

FN3K to residues to which they normally have no or difficult access. On the other hand, it is difficult to mimic the physiological intracellular milieu, which contains ligands of hemoglobin (2,3-bisphosphoglycerate), as well as systems to maintain the ATP concentration (required for FN3K) and repair other damages caused to hemoglobin (e.g. its conversion to methemoglobin).

The residues that seem to be readily deglycated as a consequence of their phosphorylation by FN3K (Lys-α-16, Lys-α-139 and Lys-β-17) in erythrocytes incubated with 200 mM glucose are not present in the profiles from erythrocytes incubated with 5 mM glucose, whereas other residues (Val-β-1, Lys-α-61) were easily detected. Glycation in the ‘5 mM glucose’ conditions mostly results from the prolonged (60 days as a mean) contact of hemoglobin in vivo with glucose before the ex vivo incubation. FN3K has therefore had ample time to phosphorylate and cause deglycation of fructosamines formed at the level of Lys-α-16, Lys-α-139, and Lys-β-17. By the same token, the fact that the peptide containing the modified Lys-β-144 is not detectable in the control profile (5 mM glucose) indicates that this residue is slowly deglycated in vivo.

Fig. 6. Three-dimensional structure of human oxy-hemoglobin showing the position of glycated residues. A space-filling rendition of the structure determined by Pauli et al. (21) is shown. In B, the model shown in A has been rotated 180° along the vertical axis and 115° counterclockwise along the plane of the figure. α and β chains are displayed in gray and white, respectively and heme groups in black. Pink residues correspond to the sites where fructosamines phosphorylated by FN3K have been identified, whereas violet residues were found to be glycated in previous studies (see Table II). Residues interacting with 2,3-bisphosphoglycerate (besides Val-β-1 and Lys-β-144, i.e. His-β-2, Lys-β-82, and His-β-143) are highlighted in green.
to conclude from our data which of the residues is DMF-sensitive and say whether Lys-β-66 is poorly deglycated in intact erythrocytes.

**Physiological Significance of Deglycation**—The physiological importance of deglycation is unknown at present. Deglycation might prevent the further conversion of fructosamines into advanced glycation products. FN3K would be expected to act on glycated residues that are most accessible and that would therefore be more likely to cause covalent cross-linking of proteins. Another potential role is to prevent the loss of residues that play a critical role in enzyme catalysis or protein function. Catalytic sites are usually buried, and it is likely that many of them would not be accessible to FN3K. However, enzymes sometimes undergo rather large conformational changes during catalysis. A glycated lysine might therefore become accessible in an open conformation of an enzyme. A third role of deglycation could be to prevent the loss of residues playing a role in protein/protein interactions. Such residues would be expected to be rather freely accessible to FN3K.

Whatever the role of protein deglycation, the findings of the present work indicate that accessibility seems to be a crucial factor in protein repair. If FN3K could be associated with a hypothetical protein unfolding system, the fructosyl residues would be expected to be deglycated with similar efficiencies. This points to a major difference of DNA repair systems, for which the modified bases are equivalently accessible (19, 20), and protein repair systems, which can only take care of the fraction of the amino acid residues that are accessible.

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