The Substitution of a Single Amino Acid Residue (Ser-116 → Asp) Alters NADP-containing Glucose-Fructose Oxidoreductase of *Zymomonas mobilis* into a Glucose Dehydrogenase with Dual Coenzyme Specificity*

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Glucose-fructose oxidoreductase (GFOR, EC 1.1.1.99.-) from the Gram-negative bacterium *Zymomonas mobilis* contains the tightly bound cofactor NADP. Based on the revision of the gfo DNA sequence, the derived GFOR sequence was aligned with enzymes catalyzing reactions with similar substrates. A novel consensus motif (AGKHVCEKPF) for a class of dehydrogenases was detected. From secondary structure analysis the serine-116 residue of GFOR was predicted as part of a Rossmann-type dinucleotide binding fold. An engineered mutant protein (S116D) was purified and shown to have lost tight cofactor binding based on (a) altered tryptophan fluorescence; (b) lack of NADP liberation through perclorlic acid treatment of the protein; and (c) lack of GFOR enzyme activity. The S116D mutant showed glucose dehydrogenase activity (3.6 ± 0.1 units/mg of protein) with both NADP and NAD as coenzymes (Kₘ for NADP, 153 ± 9 μM; for NAD, 375 ± 32 μM). The single site mutation therefore altered GFOR, which in the wild-type situation contains NADP as nondissociable redox cofactor reacting in a ping-pong type mechanism, to a dehydrogenase with dissociable NAD(P) as cosubstrate and a sequential reaction type. After prolonged preincubation of the S116D mutant protein with excess NADP (but not NAD), GFOR activity could be restored to 70 units/mg, one-third of wild-type activity, whereas glucose dehydrogenase activity decreased sharply. A second site mutant (S116D/K121A/K123Q/I124K) showed no GFOR activity even after preincubation with NADP, but it retained glucose dehydrogenase activity (4.2 ± 0.2 units/mg of protein).

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1 The abbreviations used are: GFOR, glucose-fructose oxidoreductase; kb, kilobase; MES, 4-morpholineethanesulfonic acid; HPLC, high performance liquid chromatography.
Growth in complex medium supplemented with isopropyl-1-thio-
from pZY507 as described elsewhere (6).

Site-directed Mutagenesis—Site-directed mutagenesis was carried out on a derivative of M13mp18 (16) containing an internal 0.3 kb PstI/SphI fragment of the gfo gene according to a standard procedure (17) with oligonucleotides I, 5’-GCTTTTACGGTGTTAAGCAAGCTTGAT-3’ (S116D); II, 5’-AAGCCCATGTTAAGCCGATT- TCTAGACGAGGTCAACAGGAG-3’ (S116D/K121A/K123Q/ I24K); and III, 5’-TAAATCTGTTAAGCAAGCATATTTACCGA- C-3’ (A95G); altered base triplets are underlined. In a second round of mutagenesis, an M13 clone, with the exchange A95G as used template together with oligonucleotides I and II to combine these mutations. Base exchanges were checked by DNA sequencing. After verification, the respective fragments were cloned into plasmid pZY470/S116D or pZY470/S116D/K121A/K123Q/ I124K to combine these mutations. The resulting plasmid I124K was used as template for the second round of mutagenesis according to the manufacturer’s protocols (Pharmacia LKB, Freiburg, Germany).

Detection of Bound NADP(H)—Tightly bound NADP(H) was released from GFOR by acid denaturation and was subsequently detected by HPLC analysis. The enzymes were equilibrated to sodium phosphate buffer (200 mM, pH 5.0) by ultrafiltration and diluted to a final concentration of approximately 4 mg/ml. 60 μl of ice-cold perchloric acid (35%) was added to 60 μl of the enzyme solution, mixed, and kept on ice for 15 min. The samples were neutralized by the stepwise addition of 180 μl of KHCO₃ (2 M), and insoluble material was spun down by centrifugation. An aliquot of the supernatant was submitted to HPLC on an octadeyl-silica gel column (Hypersil ODS, 5 μm, CS Chromatography Service, Langenwehe, Germany) that was eluted with a gradient of sodium phosphate (200 mM, pH 5.0), acetonitrile at a flow rate of 0.3 ml/min at 40 °C. Retention times of NADP(H) and NADH were determined with standard solutions.

Enzymatic Measurements—Enzyme activities were determined at 30 °C in a thermostatted cuvette holder of a Shimadzu UV160 spectrophotometer by the measurement of acidification (formation of gluconic acid from glucose) according to a published method (1). Gluconolactone from Rhodotorula rubra was added in excess to the reaction mixture (7). Glucose dehydrogenase activity was measured by the increase of reduced NADP (340 nm). Reaction mixtures contained 5 μg/ml purified protein (GFOR or mutant protein), 5 units/ml glucono-lactonase, glucose (400 mM in 40 mM K-MES buffer, pH 6.4), and 1 mM NADP. To determine Kₘ, concentrations of coenzymes were varied from 2 to 1,000 μM Kₘ, and standard deviations thereof were calculated by the Enzfitter Program (Elsevier Biosoftware, version 1.05). Protein was determined by a dye binding method (21).

Fluorescence Spectroscopy—Fluorescence spectroscopy was performed with an Amino-Bowman Series 2 Spectrometer at 20 °C. Prior to use, the enzyme solutions were equilibrated by ultrafiltration to sodium phosphate buffer (50 mM, pH 6.4). Excitation and emission slits were set to 4 nm. To minimize photodecomposition of the enzymes, the shutter of the exciting beam was kept closed until the measurement started. Fluorescence titrations were performed by the stepwise addition of 2.5–5 μl of NADP(H) to 2 ml of an enzyme solution with a concentration of 0.6 μM (tetramer). Dilution by the addition of NADPH

\begin{align}
(F - F_i)(F - F_0)^{-1} &= \frac{1}{2}[E]_0(K_i + [E]) + [S]
\end{align}

\begin{align}
&\pm \sqrt{(K_i + [E] + [S]) + 4[E][S]}^2
\end{align}

where [E]₀ and [S]₀ represent the initial enzyme and NADPH concentrations, respectively; \(F_i\) is the fluorescence intensity at saturating NADPH concentration; \(F_0\) is the fluorescence intensity at a given NADPH concentration without enzyme; and \(F\) is the fluorescence intensity at a given NADPH concentration with enzyme.

**Fig. 1.** Scheme of the ping-pong type reaction catalyzed by glucose-fructose oxidoreductase. NADP(H) as a redox carrier remains tightly bound to the enzyme. Gluconolactone is subsequently hydrolyzed to glacial acid either spontaneously or by a specific lactonase.

**Fig. 2.** SDS-polyacrylamide gel electrophoresis with various purified GFOR mutant proteins. Lanes 1 and 10, molecular weight markers “comithekl” (Boehringer Mannheim); lane 2, GFORA32–46; lanes 3–7, mutant proteins A–E; lanes 8 and 14, purified GFOR (wild-type); lane 9, fraction from purification of mutant protein D, containing exclusively a degradation product described in the text; lane 11, GFORA32–74, lane 12, mutant F; lane 13, mutant G. Note the faint degradation protein bands in lanes 6 and 7.
NADP Binding by Glucose-Fructose Oxidoreductase

RESULTS

Revised gfo Sequence and Prediction of the Secondary Structure of GFOR—During former rounds of subcloning and site-directed mutagenesis (6) we had already encountered several deviations from the published DNA sequence of the gfo gene (5). This prompted us to sequence the complete gfo gene again. In a comparison with the former sequence (5), several frame-shifts were observed which resulted in a deviating amino acid sequence of GFOR comprising only 433 residues (instead of 439). According to our DNA sequencing, the COOH-terminal sequence of GFOR—sequence, we performed similarity searches with the HUSAR package provided by the European Molecular Biology Laboratory (EMBL, Heidelberg) in all accessible data bases using the uncorrected GFOR sequence, have been established that the fingerprint regions of binding sites for NAD or NADP of approximately 30 amino acid residues (Fig. 3). GFOR displays the characteristic fingerprint of a NAD binding motif using site-directed mutagenesis. This prompted us to sequence the complete gfo gene again and hydrophobic; , glycine; , acid. The secondary structure elements of GFOR resulting from the PHD prediction (27) are shown as gray (β-sheet) or open (α-helix) boxes at the top of the sequences. The first α-helical region of GFOR (dotted box) results from a separate secondary structure prediction on GFOR and was not yielded with the prediction of all aligned enzymes. The processing site of the GFOR presecretory protein between amino acid residues 52 and 53 is marked by an arrow. MocA and ORF334, putative ribonucleotide dehydrogenases of Escherichia coli (24); DDP-DH, phtalic acid dehydrogenase from Pseudomonas putida (45); GalDH, galactose dehydrogenase from Pseudomonas fluorescens (46); StrI, putative protein in streptomycin biosynthesis from Streptomyces griseus (47); BabRed, biliverdin reductase from rat kidney (48); InhD, inositol dehydrogenase from B. subtilis (36); LmbZ, putative gene product of lmbZ of lincomycin biosynthesis from Streptomyces lincolnensis (49).

apparent; this box is found around amino acid residues 170–185 of GFOR. A data base search for this motif returned exclusively the sequences listed in Fig. 3. All of the listed enzymes, with the exception of biliverdin reductase, are known, or can be expected, to react with substrates that are structurally similar to glucose. Therefore this motif may constitute a putative fingerprint for a novel class of sugar dehydrogenases.

A secondary structure prediction of GFOR was performed using the PHD program (27), based on the multiple alignment shown in Fig. 3. The amino-terminal half of GFOR, according to this prediction, consists of six α-helical elements, resembling the structure of Rossmann-type NAD(P) binding sites (28). Taken together with the possible fingerprint motif for NAD(P) binding sites, it could be predicted that GFOR binds its cofactor NADP in a domain comprising the NH2-terminal half (approximately amino acid residues 80–250) in a βαβ dinucleotide binding fold, resembling the Rossmann fold of dehydrogenases.

Rationale for GFOR Mutant Protein Design—Only a few amino acid residues are highly conserved in Rossmann folds. These are 10–11 amino acid residues, termed the fingerprint sequence of bab dinucleotide binding folds, in the region of the first and second β-sheet (βa and βb) and the interspacing pyrophosphate binding α-helix (αb). From sequence and structural data and from mutational analyses, it has been established that the fingerprint regions of binding sites for NAD or NADP differ to some extent and that these differences play a key role in determining the coenzyme specificity (29–31). To analyze the mode of NADP binding in GFOR and to assess the involvement of a possible Rossmann fold, we intended to weaken the interaction of GFOR with its cofactor NADP by engineering an NAD binding motif using site-directed mutagenesis.

The conserved Ala residue in NADP binding sites is known to induce a hydrogen bond pattern that differs from that of NAD

\[
\text{GFOR} \quad \text{MVPATRPMPW} \quad \text{DRPQAYLI} \quad \text{LGKXYALQ} \quad \text{GQFQQGGRS} \quad \text{ISKLVLXNQ} \quad \text{KAIKAVAEYG} \quad \text{VDPKRYDYS} \quad \text{NDPKIADKP} \quad \text{IDAVYYLNN}
\]

\[
\text{MocA} \quad \text{MTYQPQSLQG} \quad \text{ARHGQMVVR} \quad \text{AAAACSLV} \quad \text{AAVADIAIIA} \quad \text{SRLLAGKQN} \quad \text{KD} \quad \text{YTR} \quad \text{EADVISS} \quad \text{VGYLIALTNS}
\]

\[
\text{ORF334} \quad \text{MDYVAPQRII} \quad \text{TARAAVDEII} \quad \text{DSLLGSKK} \quad \text{VLYSAISSLK} \quad \text{RARAAT} \quad \text{IRGX} \quad \text{YSYD} \quad \text{DIEPS} \quad \text{IDAVIIPLNN}
\]

\[
\text{GalDH} \quad \text{MQPRLGNY} \quad \text{GQKDAQQPI} \quad \text{R} \quad \text{R} \quad \text{A} \quad \text{AIAYNAPF} \quad \text{TLUSVQTPQ} \quad \text{PCFQGYMPQG} \quad \text{LQGILRQQP} \quad \text{VDAIAIPCTP}
\]

\[
\text{Strl} \quad \text{NPQIVW} \quad \text{AAGINGRRHAR} \quad \text{TTLELDPP} \quad \text{LVUPHGV} \quad \text{P} \quad \text{G} \quad \text{G} \quad \text{G} \quad \text{G} \quad \text{G} \quad \text{G} \quad \text{G}
\]

\[
\text{LmbZ} \quad \text{NTHRCGDD} \quad \text{TOLQATRVR} \quad \text{A} \quad \text{A} \quad \text{A} \quad \text{A} \quad \text{A} \quad \text{A} \quad \text{A}
\]
enzymes were denatured with 6 M guanidinium hydrochloride. In the denatured state, wild-type GFOR and mutant B behaved similarly to mutant B (data not shown).

To examine whether the differences in intensity of tryptophan fluorescence between wild-type GFOR and mutant B reflected differences in the native conformation, the respective proteins were denatured with 6 M guanidinium hydrochloride. In the denatured state, wild-type GFOR and mutant B showed the same fluorescence at 330 nm (Fig. 5). However, no NADPH fluorescence at 450 nm with the wild-type enzyme, with the same emission maximum at 330 nm (Fig. 5). These results indicate that mutant B did not contain any protein (data not shown). We deduce that a single amino acid residue exchange (S116D) is sufficient to destroy tight cofactor binding to GFOR.

Binding sites, where Gly occupies this position (30, 32). To assay this for GFOR, we changed Ala-95 of GFOR to Gly (A95G; mutant A, Fig. 4). Negatively charged amino acid residues (Glu or Asp) are invariably found at the end of the second β-sheet of NAD binding sites. The oxygen atoms of the side chain carboxyl group form hydrogen bonds to the 2'- and 3'-OH groups of the adenine ribose moiety of NAD. In contrast, NADP binding sites usually contain an uncharged amino acid residue at this position, which is followed immediately by a positively charged residue in many cases (29, 33). Our secondary structure predictions suggested that Ser-116 at the end of the putative β strand in GFOR might be replaced by Asp (S116D; mutant B) to lower the affinity for NADP and to combine mutations A and B to release any bound cofactor (oxidized or reduced forms of NADP or NAD), protein from wild-type GFOR was determined after preincubation of 5 mg/ml each of GFOR with 5 μg/ml enzyme <0.01/min). GFOR activities were determined with the standard photometric test without supplemented NADP (−NADP). In addition, GFOR activities were determined after preincubation of 5 mg/ml each of GFOR with NADP in 40 mM K-MES, pH 6.4, for 5 min, at 25 °C (−NADP). The final concentration of enzyme was 5 μg/ml and 20 μM NADP in the assay mix.

Site-directed mutageneses were performed as described under "Materials and Methods." The resulting mutant alleles were introduced and expressed in the GFOR-defective strain Z. mobilis ACM3963 (6, 11). These and additional mutant proteins (see below) are listed in Fig. 4. Mutant proteins were purified to apparent homogeneity as judged by SDS-polyacrylamide gel electrophoresis (Fig. 2).

The enzymatic assay for GFOR activity and Fluorescence Spectroscopy—

Specific GFOR and glucose dehydrogenase (GlcDH) activities of GFOR32–46, GFOR32–74, and mutants thereof (compare with Fig. 4) are shown. Mean values and deviations were obtained from two independently performed assays. ND, not detectable (ΔE/min for the GFOR and glucose dehydrogenase test with 5 μg/ml enzyme <0.01/min).

Table I

| Glucose-fructose oxidoreductase and glucose dehydrogenase activities |
|--------------------------|--------------------------|--------------------------|
| −NADP | +NADP | GlcDH activity |
| μmol/min/mg | μmol/min/mg | μmol/min/mg |

GFOR32–46

| Glucose-fructose oxidoreductase and glucose dehydrogenase activities |
|--------------------------|--------------------------|--------------------------|
| −NADP | +NADP | GlcDH activity |
| μmol/min/mg | μmol/min/mg | μmol/min/mg |

GFOR32–46, GFOR32–74, and mutants thereof (compare with Fig. 4) are shown. Mean values and deviations were obtained from two independently performed assays. ND, not detectable (ΔE/min for the GFOR and glucose dehydrogenase test with 5 μg/ml enzyme <0.01/min). GFOR activities were determined with the standard photometric test without supplemented NADP (−NADP). In addition, GFOR activities were determined after preincubation of 5 mg/ml each of GFOR32–46, GFOR32–74, or mutants thereof with 20 μM NADP in 40 mM K-MES, pH 6.4, for 5 min, at 25 °C (−NADP). The final concentration of enzyme was 5 μg/ml and 20 μM NADP in the assay mix.

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The Single Amino Acid Exchange S116D (Mutant B) Leads to a Mutant Enzyme Displaying Glucose Dehydrogenase Activity with Dual Coenzyme Specificity—The enzymatic assay for GFOR activity is usually performed without NADP in the reaction mixture (1), and the formation of gluconic acid is followed by the acidification of the reaction mixture using p-nitrophenol as the pH indicator. As mutants B and C (data not shown) obviously did not contain a tightly bound cofactor, we assayed the GFOR mutant proteins for glucose dehydrogenase activity. In the reaction mixtures, which contained excess NADP, formation of NADPH was followed by the increase in
absorbance at 340 nm. Indeed, in contrast to the wild-type enzyme and mutant A, mutants B and C were active as glucose dehydrogenases with apparent activities of about 3.5 units/mg of protein (Table I). NAD was also used as cosubstrate and resulted in similar glucose dehydrogenase activities. In the reverse reaction, gluconolactone and NADP were used at an apparent Vₘₐₓ of about 0.6 unit/mg of protein (data not shown), but the inherent instability of gluconolactone at the given pH prevented a more detailed study of this reaction. The mutant proteins displayed no detectable activity as fructose reductase or as sorbitol dehydrogenase when NADPH or NADH was prevented a more detailed study of this reaction. The mutant proteins displayed no detectable activity as fructose reductase or as sorbitol dehydrogenase when NADPH or NADH was present in the reaction mixtures (data not shown).

As NADP and NAD were used as cosubstrates in the glucose dehydrogenase reaction of mutants B and C, we were able to measure respective Kₘ values toward these pyridine nucleotides (Table II). Mutants B and C showed higher affinity for NADP than for NAD, judged by the lower Kₘ values for NADP. This indicated that the mutant proteins preferred the native cofactor of GFOR, NADP. The turnover number (k₅ₐₜ) and the k₅ₐₜ/Kₘ values as criteria for the overall kinetic properties showed that the dehydrogenase reaction of mutants B and C was slow and that NADP was a better substrate than NAD.

Thus, a single amino acid exchange S116D (mutant B) leads to a mutant GFOR displaying glucose dehydrogenase activity with dual coenzyme specificity for NADP and NAD.

**Mutant B (S116D) Develops GFOR Activity upon Preincubation with NADP**—Purified mutant proteins B and C have apparently lost their cofactor, and this is the reason for lack of GFOR activity. We assayed whether the mutant proteins B and C would behave like apoenzymes that could regain GFOR activity after prolonged preincubation with an excess of cofactor for an efficient restoration of enzymatic activity. Indeed, using mutant proteins B and C, GFOR activities could be restored to approximately one-third of the wild-type enzyme activity (Table I). However, when the preincubation step was omitted, and NADP was added directly to the test mixture (at the same final concentration), GFOR activity from mutant protein B could not be detected over a period of 5 min. When the preincubation step was prolonged from 5 to 30 min, GFOR activity of mutant B increased further from about 50 to about 70 units/mg (Fig. 6). Longer incubation with NADP (up to 7.5 h) did not increase GFOR activity beyond 75 units/mg. In contrast, glucose dehydrogenase activity decreased significantly over time (Fig. 6). Thus, a kinetic correlation between increasing GFOR and decreasing glucose dehydrogenase activities appeared. As glucose dehydrogenase lost its activity also in the absence of NADP in a linear manner, the enzyme was inherently unstable. We infer that mutant B undergoes a partial conformational change upon preincubation with NADP, yielding a conformation that binds NADP tightly and which exhibits GFOR activity and excludes glucose dehydrogenase activity.

**Combination of Exchange S116D with Additional Mutations Results in Complete Loss of GFOR Activity**—To analyze if a mutant enzyme with the complete loss of GFOR activity could be engineered by further reducing the affinity for NADP of mutant protein B, we performed additional exchanges of amino acid residues that were outside of the putative Rossmann fingerprint sequence of GFOR. Positively charged amino acid residues that follow directly the fingerprint motif may stabilize the 2'-phosphate group of NADP (29, 31, 33). As a blueprint for GFOR mutagenesis, we used the sequence of an NAD-dependent enzyme, inositol dehydrogenase of *Bacillus subtilis* (36). This enzyme is the only well characterized and strictly NAD-dependent enzyme (37) with striking sequence similarities to GFOR (Fig. 3). Therefore, the region around the positively charged residues Lys-121 and Lys-123 of GFOR was exchanged by respective amino acid residues (K121A/K123Q/I124K) to mimic the inositol dehydrogenase sequence in this region. This additional exchange of positively charged residues (mutant proteins D and E, Fig. 4) had no severe effect on the glucose dehydrogenase activity when compared with the respective ancestor proteins B and C (Table I). The Kₘ values for NADP are increased (Table II), which might result from the lack of interaction between Lys-121 and/or Lys-123 and the 2'-phosphate of NADP. For NAD, only a slight increase of Kₘ values was observed. More importantly, with mutant proteins D and E, no GFOR activities could be detected by the standard photometric assay. In contrast to the ancestor proteins B and C, GFOR activities could not be restored after preincubation with NADP (Table I).

**Fig. 5.** Fluorescence emission spectra of GFORΔ32–46 (solid lines) and mutant B (dotted lines) after preincubation with 400 mM glucose. Excitation was at 295 nm and emission was monitored in a range from 305 to 550 nm. The final concentration of the enzymes was 13130 cat/min per mg protein (data not shown).

**Table II**

Kinetic parameters of glucose dehydrogenase with the coenzymes NADP and NAD

| Mutant | Kₘ (μM) | k₅ₐₜ (min⁻¹) | k₅ₐₜ/Kₘ (μM⁻¹ min⁻¹) |
|--------|---------|---------------|------------------------|
| B      | 153 ± 9 | 629           | 4.1                    |
| C      | 126 ± 9 | 539           | 4.3                    |
| D      | 227 ± 27| 880           | 3.9                    |
| E      | 370 ± 30| 1,101         | 3.0                    |
| F      | 74 ± 9  | 485           | 6.6                    |
| G      | 240 ± 26| 780           | 3.3                    |

| Mutant | Kₘ (μM) | k₅ₐₜ (min⁻¹) | k₅ₐₜ/Kₘ (μM⁻¹ min⁻¹) |
|--------|---------|---------------|------------------------|
| NADP   | 375 ± 32| 1,089         | 2.9                    |
| NAD    | 455 ± 58| 1,035         | 2.3                    |
|        | 487 ± 50| 1,368         | 2.8                    |
|        | 501 ± 53| 1,068         | 2.1                    |
|        | 144 ± 17| 999           | 6.9                    |
|        | 570 ± 34| 1,319         | 2.3                    |
Evidently, the affinity of NADP(H) to mutants D and E is reduced. As a direct and sensitive method to determine the affinity of mutant GFOR to NADPH, we measured the interaction of protein with cofactor by the fluorescence enhancement of NADPH upon binding to the apoprotein. This method can be used to calculate the dissociation constant \( K_d \) of NADP(H) to various dehydrogenases (23) and is based on the fact that NADP(H) fluorescence intensity is enhanced upon specific binding to the protein. A titration curve with mutant proteins B and D relating the NADPH fluorescence intensity to the concentration of added NADPH is given in Fig. 7A. In contrast to B, no major fluorescence enhancement could be measured in the range of 0–10 \( \mu \)M NADPH for mutant D, showing that the affinity of mutant protein D for NADPH is greatly reduced. The dissociation constant \( K_d \) for mutant B was calculated by fitting the values of the titration experiment to Equation 1 (Fig. 7C). A \( K_d \) of 0.3 \( \mu \)M was derived for mutant B.

Deletion \( \Delta 2–74 \) Affects GFOR Activity but Not Glucose Dehydrogenase Activity in Combination with Exchange S116D—During purification steps, we observed that the protein stability of mutants B, C, D, and E was severely affected. After the cation-exchange chromatography step, in several fractions a smaller protein of about 38 kDa could be seen both in SDS-polyacrylamide gels (Fig. 2) and in Western blots (data not shown). From a nearly homogeneous preparation, this form of mutant D (lane 9 of Fig. 2) was shown to be active as glucose dehydrogenase. Using limited Edman degradation, the NH2-terminal amino acid residues were determined to be Ile-Arg-Pro-Met-Pro, which match the amino acid residues from position 75 to 79 of GFOR. Thus, this smaller protein is a degradation form of mutant D resulting from proteolytic truncation at its NH2 terminus. Degradation had removed a proline-rich sequence while retaining the putative Rossmann fold.

The calculated molecular mass of the truncated protein of 40,027 Da was in good correlation to the size estimated by SDS-polyacrylamide gel electrophoresis (about 38 kDa, Fig. 2).

As the NH2-terminal truncation apparently only occurred when tight binding of NADP was affected (mutants B–E), we examined whether the proline-rich region from position 53 to 74 of GFOR fulfilled a specific function in the tight binding of NADP (amino acid residues 2–52 represent the signal sequence that is normally processed during export to the periplasm). The coding region for amino acid residues 2–74 in the plasmid-encoded gfo gene was deleted by a polymerase chain reaction method (18). In addition, this deletion was combined with mutation B or D. The resulting alleles \( \Delta 2–74 \), F and G (Fig. 4) were expressed in Z. mobilis ACM3963, and the mutant proteins were purified to apparent homogeneity (Fig. 2).

With mutant GFOR\( \Delta 2–74 \), neither GFOR nor glucose dehy-
droughtenase activity could be measured (Table I), nor could GFOR activity be restored upon preincubation with NADP. With mutants F and G, glucose dehydrogenase activity could be detected at similar levels as with mutants B–E. Again, no GFOR activity was detected no matter whether the protein was preincubated with NADP or not (Table I). $K_m$ values for mutant F (S116D combined with $\Delta^2-74$) for NADP and NAD were decreased by a factor of approximately 2 compared with mutant B (S116D). For mutant G, no severe change of $K_m$ for NADP and NAD was measured compared with the respective mutant E without NH$_2$-terminal truncation. In fluorescence spectra, mutant proteins $\Delta^2-74$, F, and G showed the same enhanced tryptophan fluorescence as mutant B, indicating that no NADP(H) was bound to the proteins. No NADP(H) was detected in supernatants of GFOR$\Delta^2-74$ denatured with perchloric acid (data not shown). The affinity to NADPH of mutant proteins GFOR$\Delta^2-74$, F, and G was measured by fluorescence titration (Fig. 7B). NADPH fluorescence enhancement with GFOR$\Delta^2-74$ ascended steeper than the titration curve of mutant F, indicating a higher affinity for NADPH (38).

When fitted to Equation 1, a $K_f$ of 0.3 $\mu$m for mutant F was calculated (Fig. 7C) under the assumption that four NADP binding sites/enzyme are present (1; inset in Fig. 7C). For GFOR$\Delta^2-74$, a $K_f$ of 0.04 $\mu$m was determined. However, for GFOR$\Delta^2-74$ only two NADP binding sites were calculated (inset in Fig. 7C). The reason for this discrepancy is unknown. Mutant F showed only slight fluorescence enhancement with NADPH concentrations up to 10 $\mu$m. These data showed that mutant GFOR$\Delta^2-74$ was able to bind NADP with a higher affinity than mutants E or F. The lack of GFOR and glucose dehydrogenase activity in mutant protein GFOR$\Delta^2-74$ is therefore not due to a mere defect in NADP(H) binding. As the same $K_f$ values for NADPH were obtained for mutant proteins B and F, it may be inferred that the proline-rich region of wild-type GFOR has no direct effect on the affinity for NADP(H).

**DISCUSSION**

Based on the revised amino acid sequence derived of the gfo gene and from our mutational analyses it appears that the cofactor NADP binds to the $\beta\beta\beta$-dinucleotide binding motif of GFOR (Rossmann fold; 28). To probe this suggestion and to analyze the mode of tight NADP binding in GFOR, we used site-directed mutagenesis in the region of the fingerprint motif with the intention of weakening the interaction of the GFOR protein with its cofactor NADP. We reasoned that a suitable way to accomplish this was to introduce an NAD binding motif. The exchange A95G had resulted in a 40-fold decrease of the terms of tight cofactor binding or specificity. In the NADP-dependent effect on the NADP binding characteristics, as no difference was observed when GFOR had been treated with or without NADP (Table I). However, when preincubated with high concentrations of NADP concomitant with the decrease in glucose dehydrogenase activity which was not found with wild-type GFOR. It may be concluded that the overall structure of the dinucleotide binding fold is maintained in mutant B and that the lower affinity for NADP allowed the free exchange of bound NADP with soluble NADP. The cofactor NADP was thus changed to a cosubstrate, and the oxidation of glucose was separated from the reduction of fructose. Therefore, the single site mutation altered GFOR to a dehydrogenase with dissociable NADP as cosubstrate and a sequential reaction type, in contrast to the wild-type enzyme, which reacts in a ping-pong type mechanism and contains NADP as a nondissociable redox cofactor. Interestingly, mutant B acts neither as fructose reductase nor as sorbitol dehydrogenase, although from kinetic studies on GFOR it has been postulated that glucose and fructose occupy the same binding site (41). It may be that additional conformational requirements for the binding and/or turnover of fructose can only be adopted when NADP is tightly bound.

The dual coenzyme specificity of the glucose dehydrogenase reaction for NADP and NAD of mutant B underlines the key role of residue Ser-116 for cofactor recognition. These results are in good agreement with an analogous mutation in the NADP-dependent cinnamyl-alcohol dehydrogenase isomerase of Eucalyptus gunnii (42), where a Ser residue was also involved in recognition of the 2'-phosphate of NADP. There, a single exchange S212D had resulted in a 2.2 $\times$ 10$^3$-fold decrease of the overall catalytic efficiency for NADP, whereas this parameter had not been significantly changed for NAD.

To our knowledge, this is the first report that an enzyme catalyzing a so-called complex pyridine nucleotide-dependent transformation (43) is changed to a dehydrogenase by site-directed mutagenesis. We suggest that restoration of GFOR activity in mutant B upon preincubation with high concentrations of NADP concomitant with the decrease in glucose dehydrogenase activity indicates that in mutant B two possible conformations can be adopted, one with tightly bound NADP as cofactor acting as GFOR, and another with lower affinity to NAD acting as glucose dehydrogenase. The exchange of several amino acid residues (in addition to S116D) which are adjacent to the putative $\beta\beta$-sheet (the co-loop region) of GFOR by respective residues of the NADP-dependent inositol dehydrogenase resulted in a mutant (D) which was not able to restore GFOR activity but whose kinetic properties as glucose dehydrogenase almost remained the same as those of mutant B. However, fluorescence titrations showed that the affinity to NADP, compared with mutant B, was severely reduced, as little fluorescence enhancement could be detected. Therefore, residues Lys-121 and/or Lys-123 may indeed contribute to tight NADP binding, most likely by interaction with the 2'-phosphate of NADP. Such electrostatic interactions have recently been shown to be essential in NADP recognition by glucose-6-phosphate dehydrogenase, as the single exchange of Arg-46 to Gln or Ala resulted in mutant enzymes that preferred NAD (31).
From the analysis of NADP affinity in mutant proteins B and E, it may be inferred that tight binding of NADP in GFOR is achieved solely by an extension of protein-ligand interactions in the \( \beta \beta \) dinucleotide binding fold, as has been reported for the UDP-galactose epimerase of \( E. \) coli (44). However, the nature of the conformational change in mutant B necessary to restore GFOR activity is still unclear, and it is likely that additional interactions with the cofactor are produced which lock NADP in its binding site, accomplishing tight binding despite the relaxation caused in mutant S116D.

A possible explanation may be drawn from the analysis of mutant proteins \( \Delta \Delta -74 \), and derivatives thereof, which mimic the \( \mathrm{NH}_2 \)-terminal degradation product that invariably had appeared during protein purification of mutants B and D in which tight NADP binding was affected. Although \( \Delta \Delta -74 \) was not able to bind NADP(H) in the same tight manner as wild-type GFOR, fluorescence titrations clearly showed that GFOR\( \Delta \Delta -74 \) was still able to bind NADPH with high affinity. Thus, glycine dehydrogenase may be excluded as it relies on cosubstrate diffusion. Therefore, the proline-rich region preceding the \( \beta \beta \) binding fold in GFOR seems to contribute also to tight NADP binding. As, after preincubation with NADP, no GFOR activity was measured with GFOR\( \Delta \Delta -74 \), the proline-rich region is essential for the adaption of a conformation displaying GFOR activity. Mutant F exhibited glucose dehydrogenase activity but, in contrast to mutant B, did not restore GFOR activity. The proline-rich region of GFOR therefore might constitute a flexible loop. After binding of NADP to the high affinity \( \beta \beta \)-NADP binding fold of GFOR, a conformational change may induce this loop to cover the dinucleotide binding fold, thus completing tight NADP binding and creating structural requirements for GFOR activity. This hypothesis of a flexible loop is supported by the fact that truncation of GFOR was only observed when tight NADP binding was affected. It is likely that the proline-rich NH\( _2 \)-terminal loop becomes accessible to proteases when the cofactor binding site of GFOR is not occupied by NADP.

We have demonstrated by site-directed mutagenesis of Ser-116 residue (S116D) two new phenomena in GFOR: loss of tight binding of NADP cofactor and acquisition of a new enzyme activity (glucose dehydrogenase) with dual cofactor specificity. Additional mutagenesis (mutants D and E) or NH\( _2 \)-terminal deletions led to the loss of GFOR activity while glucose dehydrogenase activity was retained in the case of mutants D, E, F, and G. GFOR thus may have evolved originally from a glucose dehydrogenase-like ancestor. This is likely as the enzyme still shows sequence similarity to a class of sugar dehydrogenases and because of the behavior of some of the mutants described in this report.

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Addendum—While the present manuscript was under review, the three-dimensional structure of GFOR with its cofactor NADP was published (50). Data therein showed that GFOR, indeed, binds its cofactor by an extension of protein-ligand interactions in a typical Rossmann fold with residues Gly-90, Gly-92, and Ala-95 (our enumeration) as part of the fingerprint region. With respect to our mutagenesis approach, it is remarkable that amino acid residues Ser-116 and Lys-121 from one subunit and Arg-69 from the NH\( _2 \)-terminal arm of an adjacent subunit cooperate in forming hydrogen bonds to the 2’-phosphate of NADP. These structural data are in full accord with our mutagenesis studies.