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Control of the Association State of Tetrameric Glucose-Fructose Oxidoreductase from *Zymomonas mobilis* as the Rationale for Stabilization of the Enzyme in Biochemical Reactors[1]

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Tetrameric, NADP-containing glucose-fructose oxidoreductase (GFOR) from *Zymomonas mobilis* catalyzes the oxidation of glucose into glucono-δ-lactone coupled to the reduction of fructose to sorbitol. GFOR is inactivated during substrate turnover *in vitro*, the long-term stability of the enzyme during conversions in biochemical reactors thereby being drastically reduced. The process of inactivation is triggered by structural transitions that are induced by the lactone product and involves aggregation as the ultimate cause of irreversible inactivation. Guanidinium hydrochloride-induced changes in the conformation of GFOR seem to be similar to those observed in the presence of lactone, and are manifested by incubation time-dependent increases in protein fluorescence and the solvent-exposed hydrophobic surface. The formation of high-order protein associates in solution in the presence of this denaturant proceeds from the native tetramer to a reversibly inactivated octamer and then to a dodecameric form that cannot be reactivated through spontaneous or assisted refolding. Therefore, stabilization of GFOR during turnover requires that the marked tendency of the enzyme to form aggregates is prevented efficiently. This goal has been accomplished in the presence of low urea concentrations (1.0 M), which led to a 10-fold increase in the half-life of GFOR under operational conditions.

Key words: aggregate formation, glucose-fructose oxidoreductase, inactivation, renaturation, stabilization, *Zymomonas mobilis*.

Glucose-fructose oxidoreductase (GFOR) is a periplasmic enzyme of the ethanol-producing bacterium *Zymomonas mobilis* (*1*). The complete catalytic cycle of GFOR consists of two half reactions: the reduction of fructose to sorbitol coupled to the oxidation of glucose to glucono-δ-lactone (*2*). While *in vivo* glucono-δ-lactone is further converted into ethanol, sorbitol is not metabolized by *Z. mobilis*. By synthesizing intracellular sorbitol as an osmoregulatory solute, GFOR is thought to enable *Z. mobilis* to grow in concentrated sugar media (*3*). Use of GFOR as an isolated enzyme *in vitro* (*4-6*) or in the form of permeabilized *Z. mobilis* cells (*7-9*) provides a biocatalytic process for the production of gluconic acid and sorbitol, two compounds with many applications in the food and chemical industries.

GFOR is a tetrameric protein composed of identical 40-kDa protomers, and contains one NADP molecule bound tightly but noncovalently bound to each protein subunit (*1*). During catalysis, the nucleotide cycles between its oxidized and reduced forms, hydrogen equivalents thus being transferred from glucose to fructose (*10, 11*). The implication of the non-dissociably bound nucleotide for biocatalysis is that expensive and labile NADP need not be added exogenously in the process. The quarternary structural organization of GFOR and the mode of NADP binding are interesting. Each protomer contains an extended N-terminal arm (residues 1-31) that, by wrapping around the adjacent protein subunit, (i) stabilizes the protein tetramer and (ii) prevents dissociation of the NADP (*1*). From the results of crystallographic analysis, a putative role of the N-terminal arm in the correct oligomerization of GFOR has been proposed (*1*).

We have shown recently that GFOR is irreversibly inactivated during substrate turnover *in vitro* (*4, 5, 12, 13*). The inactivation leads to a drastic decrease in the total turnover number for the substrate and hampers the application of the isolated enzyme for the conversion of glucose/fructose mixtures into gluconic acid and sorbitol. The mechanism of inactivation is triggered by binding of the lactone product and involves several steps in series including structural transitions at the NADP site of GFOR (*12*). The structural data (*1*) suggest that conformational changes at the nucleotide site will affect the N-terminal arm of the adjacent protein subunit. It is thus interesting to note that the irreversible loss of activity is determined by the formation of high-order associates from the protein tetramer (*12*).

To better understand the aggregation behavior of GFOR

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Abbreviations: GFOR, glucose-fructose oxidoreductase [EC 1.1.99.28]; ANS, 8-anilinonaphthalene-1-sulfonic acid; GdmHCl, guanidinium hydrochloride.

and to develop a rationally devised means of stabilization of the enzyme, we have examined in this work how the destabilizing lactone product during turnover, and denaturants such as guanidinium hydrochloride (GdmHCl) and urea affect the activity, conformational properties and association state of GFOR. The results reveal that GdmHCl induces structural transitions that seem comparable to those observed during substrate turnover, and that inevitably lead to the time-dependent formation of high-order associates and consequently inactivation. The aggregation in solution proceeds sequentially from the native tetramer to an octameric and then to a dodecameric form. Only the protein octamer of GFOR can be reactivated. In contrast to GdmHCl, structural transitions induced by urea are fully reversible and do not lead to aggregation of GFOR. Urea has thus been used to *prevent* the formation of high-order associates and to stabilize GFOR activity during turnover.

EXPERIMENTAL PROCEDURES

Enzyme Production and Purification—GFOR from *Z. mobilis* spp. *mobilis* DSM 473 (DSM, Braunschweig, Germany) was produced and purified to apparent spectral and electrophoretic homogeneity as described recently (*12*). The enzyme isolated exclusively represents the fully processed, mature form of GFOR, that has a subunit size of approximately 40 kDa. For overexpression of the gene encoding gluconolactonase from *Z. mobilis*, the *Escherichia coli* DH5. α strain harboring plasmid pZKL1 was employed (*14*). The gluconolactonase was used in a partially pure form (*12*). The *E. coli* chaperonins, GroEL and GroES, were produced and isolated by reported procedures (*15, 16*).

Assays—GFOR activity was determined by a coupled assay at 25°C in the presence of excess (8–10 U·ml^{-1}) gluconolactonase. The decrease in absorbance at 405 nm of 0.13 mM *p*-nitrophenol is proportional to the amount of gluconic acid produced (*2*). One unit of GFOR activity refers to 1 μmol gluconic acid produced per minute. Protein was measured by the dye-binding assay with BSA as a standard.

Effects of Denaturants—Unless otherwise stated, the buffer was 10 mM K-Mes, pH 6.4. GFOR (0.2–2.5 mg·ml^{-1} in a total volume of approximately 5 ml) was incubated at 4°C for up to 24 h in the presence of different concentrations of GdmHCl (99.5% pure; Sigma) or urea. At regular intervals, residual GFOR activity and turbidity (aggregation) in 500-μl aliquots withdrawn from the reaction mixtures were measured. For measurement of intrinsic protein fluorescence, 50 μl of each sample was diluted 10-fold, and then fluorescence emission was recorded immediately. The binding of 8-anilinonaphthalene-1-sulfonic acid (ANS) to hydrophobic protein surfaces was determined by mixing 350 μl of a diluted sample with 20 μl of an ANS solution (1.0 mg·ml^{-1}). To allow better comparison, all fluorescence intensities (see below) are expressed relative to the concentration of soluble protein present in each sample. To remove the denaturant to study the refolding of GFOR, 250 μl of each sample was gel filtered on Pharmacia NAP5 columns equilibrated with the refolding buffer (10 mM K-Mes buffer, pH 6.4, with 10 mM $MgCl_2$, 7 mM KCl, and 1 mM dithiothreitol). The dead-time for gel filtration was approximately 5 min. Reactivation of GFOR (0.1–1.2 mg·ml^{-1}) freed of the denaturant was followed at 25°C from 0.1 up to 24 h reaction time. Assisted renaturation of GFOR was carried out in the presence of fivefold molar excesses of GroEL (14-mer) and GroES (7-mer) in the refolding buffer. ATP was added as indicated. Each experiment was carried out in triplicate, and the mean values are given.

Conformational Changes and Inactivation during Substrate Turnover—A stirred substrate solution (20 ml; 20 mM P_i, pH 6.2) containing 0.5 M each D-fructose and D-glucose was incubated in the presence of 0.5 μM GFOR at 30°C. The pH was controlled automatically by titration of the gluconic acid with 2 M Tris. To avoid depletion of the substrates, glucose and fructose were added together with the alkaline component so that both the pH and the substrate concentration remained constant. At different times, 350 μl-samples were taken, of which approximately 100 μl was heat-inactivated (10 min; boiling water bath) and used for HPLC analysis. The remainder of the original sample was immediately used for the measurement of residual GFOR activity as well as for further analyses by spectroscopic methods. GFOR activity was always corrected with the corresponding dilution factor (*cf. Analytical* methods section).

Spectroscopic Measurements—Protein aggregation was determined from measurements of the absorbance at 600 nm and light scattering with excitation and emission wavelengths of 500 nm. Both measurements gave consistent values. Fluorescence was measured with a Model F 2000 Spectrofluorimeter (Hitachi, Tokyo) at 25 ± 1°C using a slit width of 5 nm for both the excitation and emission wavelengths. Intrinsic protein fluorescence was recorded at 335 nm following excitation at 280 nm. The binding of the hydrophobic probe, ANS, to protein surfaces was determined at an emission wavelength of 470 nm after excitation at 390 nm.

Association States of GFOR—The association states of native and GdmHCl-treated GFOR were determined by gel permeation chromatography on a Pharmacia Superose-12 H10/30 column (flow rate of 1 ml·min^{-1} at 4°C). Approximately 50 μg protein was applied to the column equilibrated with 0.1 M K-Mes buffer, pH 7.3, with or without GdmHCl and 0.5 M NaCl. Calibration of the column was carried out using molecular mass standards of 40 to 670 kDa, and detection of the eluted proteins was performed at 280 nm.

Analytical—The concentrations of substrates and products were determined by HPLC using an Aminex HPX-87 C column (BioRad, Hercules, CA, USA; 7.8 × 300 mm), operated at 85°C. The eluent was 10 mM calcium nitrate at the flow rate of 0.7 ml·min^{-1}, and refractive index detection was used. The dilution of the reaction mixture because of alkali addition during turnover was accounted for by means of the mass balance of substrates and products.

RESULTS

Conformational Transitions and Inactivation of GFOR during Substrate Turnover—Our recent analysis showed that the binding of the lactone product induces a conformational change at the NADP site of GFOR (*12*). Deactivation of the cysteinyl residue that is exposed as a result of this structural transition triggers a sequential off-reaction

that leads to the formation of inactive and insoluble protein aggregates. Here, we have reassessed the inactivation of GFOR during turnover using low substrate concentrations (0.5 M fructose and 0.5 M glucose) because high fructose concentrations ($\geq$1.5 M) were found to stabilize GFOR. Measurements of the intrinsic protein fluorescence as well as ANS binding to hydrophobic protein surfaces were used as probes to detect structural changes in GFOR. Time-dependent conformational transitions were observed for GFOR that are manifested by significant increases in fluorescence amplitudes (Fig. 1) and, for protein fluorescence emission, by a 20-nm red shift of the wavelength of maximum fluorescence emission (λ_{max}). It has recently been shown that the intrinsic protein fluorescence of GFOR is entirely due to of tryptophanyl residues (*12*). The spectral shift of λ_{max} is indicative of an environmental change for tryptophans involving an increase in polarity (*17*). At the time when the exposure of the hydrophobic surface reached the maximum value, protein aggregation became significant and increased with further reaction time. The loss of enzyme activity was irreversible and correlated kinetically with the aggregation (not shown). Compared with the results of previous analysis carried out with a fructose concentration of 1.5 M (*12*), the conformational changes of GFOR during turnover occurred significantly faster when low fructose concentrations were employed.

Time-Dependent Structural Transitions and Inactivation of GFOR Induced by GdmHCl—Conformational transitions induced by GdmHCl were compared with those observed during substrate turnover. Initial experiments showed that GFOR underwent time-dependent, irreversible structural changes involving aggregation in the presence of all denaturant concentrations ranging from 1.0 and 6.0 M. Typical time courses for protein fluorescence intensity, surface hydrophobicity and aggregate formation in the presence of 2.5 M GdmHCl are shown in Fig. 2, A and B. When GFOR was added to a solution containing a denaturant, an immediate shift of λ_{max} from 315 to 335 nm was observed. The λ_{max} value did not change with further incubation time. Comparable to the results obtained for substrate turnover (Fig. 1), the evolutions of protein fluorescence and surface hydrophobicity occurred in parallel and passed through maximum values for the fluorescence intensities after approximately 100 min incubation time. As judged on comparison of the tryptophan fluorescence intensities of native GFOR and GFOR in the presence of GdmHCl, the conformational changes induced by the denaturant seem not to be very large. The time dependencies of the changes in (i) the conformational properties of GFOR (Fig. 2A) and (ii) protein aggregation (Fig. 2B) are quite similar. However, compared to the evolution of surface hydrophobicity, the incubation time corresponding to maximal aggregation of GFOR clearly shifted to higher values ($\geq$250 min). The renaturation competence of GFOR as a function of incubation time in the presence of GdmHCl was interesting. With 2.5 M GdmHCl, GFOR was inactivated rapidly, less than 10% residual enzyme activity being detectable after 10 min incubation time. When after this period of incubation a renaturation reaction was carried out, specific enzyme activity being recovered was significantly higher (30$\pm$10%) than the original level. This high "refolding" competence of inactivated GFOR was lost in a time-dependent manner during incubation in the presence of GdmHCl (Fig. 2B). At the time when aggregation was the maximum almost no active enzyme could be

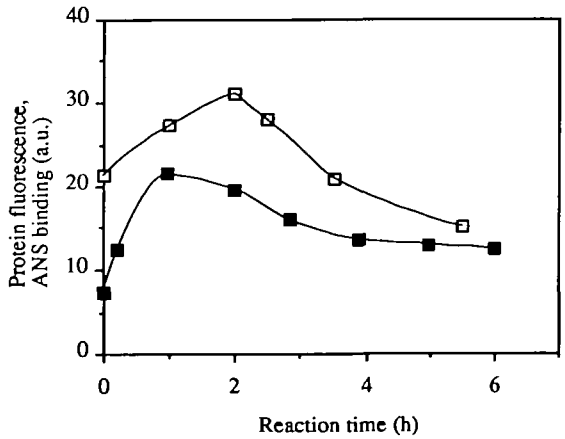

Fig. 1. **Time-dependent conformational changes of GFOR during substrate turnover.** The concentration of GFOR was 0.1 mg·ml^{-1}. A constant substrate concentration of 0.5 M was used. The pH and temperature were 6.2 (controlled by titration with 2 M Tris) and 30°C, respectively. Conformational transitions were determined as to intrinsic protein fluorescence (□) and ANS binding (■). a.u., arbitrary units.

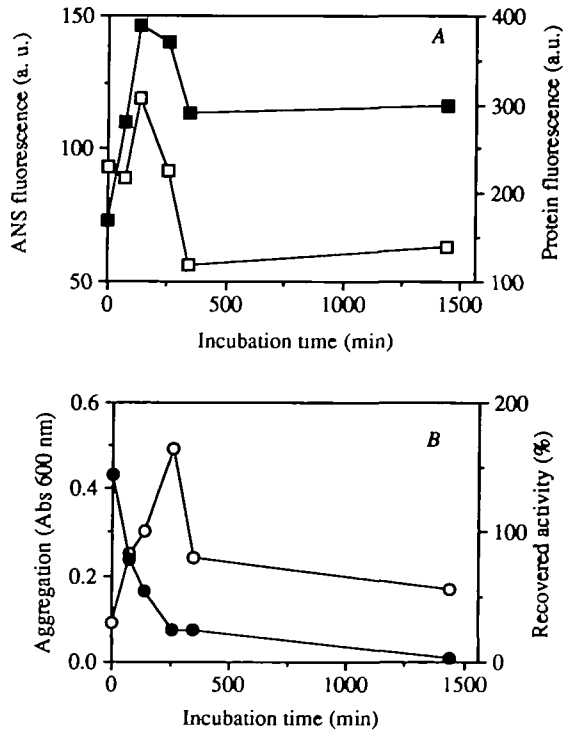

Fig. 2. **Time-dependent effects of GdmHCl on the conformational properties and renaturation competence of GFOR.** The concentration of GFOR (4°C) was 0.5 mg·ml^{-1}, and that of the denaturant 2.5 M. During refolding (25°C), the protein concentration was 0.25 mg·ml^{-1}. A: Conformational transitions of GFOR represented by changes in intrinsic protein fluorescence (□) and ANS binding (■). B: Protein aggregation (○) and spontaneous refolding capacity (●). For details see under "EXPERIMENTAL PROCEDURES." a.u., arbitrary units.

recovered. Measurement of the nucleotide fluorescence (excitation 350 nm and emission 460 nm) of gel filtered protein samples (in the presence of glucose) provided qualitative evidence that NADPH remained bound to GFOR during the GdmHCl-induced changes in conformation (not shown).

GdmHCl Concentration Dependence of Structural Transitions—The analysis of GdmHCl concentration-dependent structural transitions in GFOR is complicated by the marked time dependence of these transitions. Here, the results of 2-h incubation with denaturant at 4°C are shown. In Fig. 3, increases in protein fluorescence and the solvent-exposed hydrophobic surface of GFOR with increasing concentrations of GdmHCl are clearly visible. Whereas the exposure of the hydrophobic protein surface was constant in the range of 2.0–6.0 M GdmHCl, a critical conformational transition between 3.0 and 4.0 M GdmHCl was detectable on intrinsic fluorescence measurement. Most probably because of this transition, no GFOR activity could be recovered when denaturation was carried out with 4.0 M GdmHCl. The total protein concentration during renaturation was found not to be important in the range between 0.10 and 1.2 mg·ml^{-1}.

Subunit Organization of GFOR in the Presence of GdmHCl—The association state of soluble GFOR after 2 h incubation in the presence of 2.5 and 4.0 M GdmHCl was determined by gel permeation chromatography (Fig. 4), and compared with that of the native enzyme (180±10 kDa). In each case, GFOR was eluted as a well-defined, single protein peak, so the analysis was not complicated by the coexistence of various oligomeric protein forms in a complex mixture. The soluble form of GFOR comprises an octamer (370±20 kDa) and a dodecamer (550±20 kDa) for GdmHCl concentrations of 2.5 and 4.0 M, respectively (Fig. 4). Hence, the formation of high-order associates in solution proceeds sequentially. Like in the case of lactone-induced inactivation (*12*), structurally perturbed tetramers, but not individual protomers, seem to represent the protein unit that is prone to aggregation. Oligomeric forms of GFOR larger than the dodecamer are most likely insoluble. In the case of inactivation during turnover (Fig. 1), the soluble protein fraction corresponded exclusively to the GFOR tetramer (not shown).

Spontaneous and Assisted Refolding of Different Association States of GFOR—After denaturation in 2.5 and 4.0 M GdmHCl for 2 h, the rates of renaturation and the maximum yields of recovered GFOR activity after exhaustive renaturation were measured for different refolding conditions (Fig. 5). The enzyme concentration during renaturation was 0.1 mg·ml^{-1}. Spontaneous refolding in buffer gave 90±5% and no recovery of the original GFOR activity on previous incubation with 2.5 and 4.0 M denaturant, respectively. Hence, the octameric but not the dodecameric form of GFOR can be reactivated in a spontaneous manner. Recovery of enzyme activity from the octameric form was slow and required at least 2 h for apparent completion. When renaturation of GFOR was carried out in the pres-

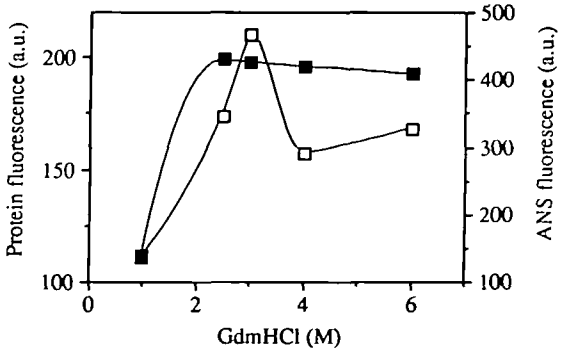

Fig. 3. **GdmHCl concentration-dependent conformational changes of GFOR.** The concentration of GFOR during incubation in the presence of GdmHCl was 0.5 mg·ml^{-1}, and the incubation time was 2 h. Changes in intrinsic protein fluorescence (□) and ANS binding (■) are shown. a.u., arbitrary units.

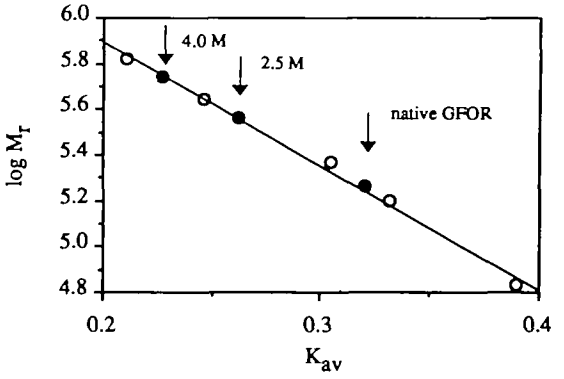

Fig. 4. **Analysis of the association state of GFOR following incubation in GdmHCl.** The relative molecular weight (M_r) of GFOR was determined by gel filtration at 4°C on Superose 12 HR 10/30. The parameter, K_{av}, was determined according to $(V_e - V_0)/(V_t - V_0)$, where V_e, V_0, and V_t are the elution volumes of one protein of known M_r, blue dextran, and acetone, respectively. Calibration (○) was carried out using BSA (68,000), aldolase (158,000), catalase (232,000), ferritin (440,000), and thyreoglobulin (669,000). GFOR (0.2 mg·ml^{-1}) was incubated at 4°C for 1.45 h in 20 mM K-Mes, pH 6.4, containing 0, 2.5, or 4.0 M GdmHCl, and then the association state of GFOR (●) was determined. The average elution time was 15 min, so the total time in the presence of the denaturant was about 2 h.

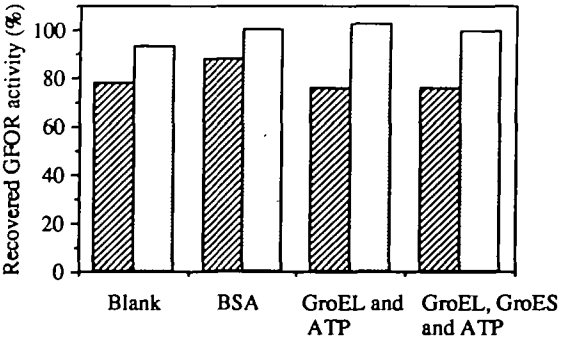

Fig. 5. **Spontaneous and assisted renaturation of an inactive GFOR octamer.** GFOR (0.2 mg·ml^{-1}) was incubated at 4°C in 2.5 M GdmHCl for 2 h. Renaturation was performed at 25°C using an enzyme concentration of 0.1 mg·ml^{-1}. The renaturation blank contained the refolding buffer and GFOR only. GroEL (14-mer) and GroES (7-mer) were added at fivefold molar excesses over GFOR. The concentration of ATP was 2 mM, and that of BSA equalled the protein concentration of GroEL plus GroES in the assay. The hatched and punctured bars show the recovered enzyme activity as percentages of the original level after 1 and 23 h, respectively.

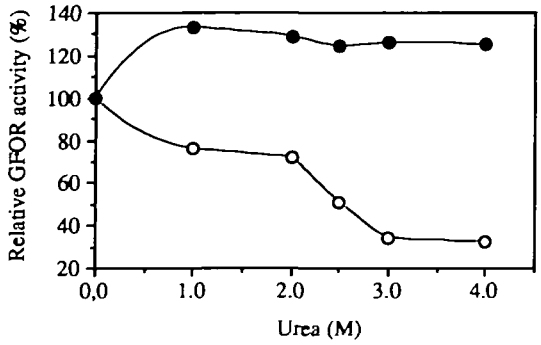

Fig. 6. **Urea-dependent inactivation of GFOR and recovery of enzyme activity.** The concentration of GFOR during incubation (4 h at 4°C) in the presence of urea was 0.5 mg·ml^{-1}, and that during renaturation 0.1 mg·ml^{-1}. Inactivation of GFOR in urea (○), and GFOR activity recovered on renaturation (●).

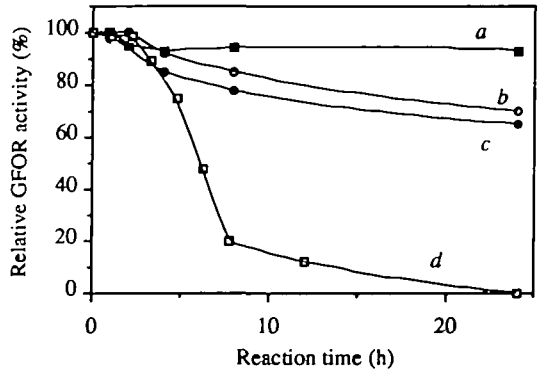

Fig. 7. **Stability of GFOR during substrate turnover and stabilization of enzyme activity on prevention of aggregation.** Experiments were carried out at 30°C and pH 6.2 using a substrate concentration of 1.5 M and a GFOR activity level of 3 U·ml^{-1}. The curves are: (a) 1.0 M urea and 10 mM DTT, (b) 10 mM DTT, (c) 1.0 M urea, and (d) control.

ence of the chaperonin system (GroEL and GroES) from *E. coli*, in the presence or absence of ATP (2 mM), the rates and yields of recovery of enzyme activity were identical within statistical significance to those of spontaneous refolding (Fig. 5). When NADP or NADPH was added to the refolding assays at concentrations between 0.1-1.0 mM, no effect on GFOR renaturation was observed.

Inactivation of GFOR Induced by Urea—A biphasic dependence of enzyme activity on the denaturant concentration was observed when GFOR was incubated in the presence of urea (Fig. 6). GFOR retained approximately 80% activity with 0.5-2.0 M denaturant. At higher concentrations of urea (up to 4.0 M), the activity decreased to a limit value of approximately 25%. Inactivation of GFOR in urea did not lead to protein aggregation detectable on light scattering or absorbance measurement, and was therefore fully reversible at all denaturant concentrations between 0.5 and 4.0 M. The specific enzyme activity recovered after treatment with urea and subsequent renaturation in the refolding buffer was even higher than the original level. The effect was significant and did not depend on the urea concentration used during the inactivation step (Fig. 6). Compared with recovery of GFOR activity from the octameric protein form, recovery of the active enzyme from any urea-inactivated state (Fig. 6) was very fast and occurred within the dead-time of the experiment (approximately 5 min for gel filtration). The stability of GFOR at 30°C in the presence of 1.0 M urea was not affected during an incubation time of 48 h, relative to the control containing no denaturant.

Stabilization of GFOR during Substrate Turnover—The effect of GdmHCl on the association state of GFOR suggests that protection against the earliest step in the aggregation process, *i.e.* formation of the octameric form, will stabilize the enzyme activity. From the results in Fig. 6 it was suspected that urea could possibly act as an anti-aggregation reagent when used during substrate turnover by GFOR. In the presence of 1.0 M urea, drastically higher, at least 10-fold, stability of GFOR was indeed found during fed-batch conversion of 1.5 M glucose and fructose, compared to in a control reaction that involved no urea (Fig. 7). Other compounds that might be capable of preventing the aggregation of GFOR, such as nonionic detergents (0.1-1.0%, by weight) or polyethylene glycol 2000-8000 (0.1%, by weight), had no stabilizing effect on the enzyme activity. In agreement with the model of product-induced inactivation of GFOR (*12*), thiol protection was important even when urea was present as a stabilizer. Hence, the highest operational stability of GFOR was observed when 10 mM DTT and 1.0 M urea were added to the reaction mixture at the same time (Fig. 7).

DISCUSSION

GFOR converts glucose and fructose into the value-added products, gluconic acid and sorbitol, and has therefore been considered as a biocatalyst with significant industrial potential. Concerning reaction engineering, advantages of GFOR are its high specific enzyme activity, its moderate inhibition by endproducts and a catalytic competence that does not rely on the presence of the soluble coenzyme, NADP. The major disadvantage of GFOR, however, is its moderate stability under operational conditions, *i.e.*, during substrate conversion. Inactivation, triggered by interactions with the glucono-δ-lactone product (*12*), severely reduces the total turnover number for each GFOR molecule *in vitro* (*4, 5*), therefore hampers efficient use of the isolated enzyme in biochemical reactors. With the aim of developing a rationally designed strategy for stabilizing GFOR, we have compared enzyme inactivation induced by the lactone product and denaturants such as GdmHCl and urea. The results showed that overall structural transitions observed for GFOR in the presence of lactone and GdmHCl (but not urea) are similar and incubation time-dependent. In contrast to product-induced inactivation (*12*), however, thiol protection by 1 mM DTT did not lead to stabilization as to inactivation in GdmHCl. Conformational destabilization of GFOR always led to aggregation of the protein tetramer. Because aggregation determines the loss of enzyme activity, stabilization of GFOR requires that the tendency to form high-order associates is prevented efficiently.

The aggregation process of GFOR exhibited several interesting aspects pertaining to its apparent specificity and reversibility. First of all, conformational changes observed during the course of inactivation are small, but trigger

incorrect association even with low protein concentrations. For example, the maximal increase in the hydrophobic surface for GFOR is only approximately 2-fold when the enzyme is incubated in the presence of 2.5 M GdmHCl or a substrate solution containing 0.5 M each glucose and fructose. In the cases of several other proteins, *e.g.*, subtilisin (*18*) and barstar (*19*), there is a large, at least 8-10-fold, difference in the exposed hydrophobic surface for the native and non-native protein forms. Second, in the presence of GdmHCl, the oligomerization of GFOR proceeds in discrete steps from the native tetramer to the inactive octameric and then to the dodecameric form. In the case of inactivation during turnover, the reactions of protein association and subsequent precipitation are difficult to separate kinetically, so the only detectable soluble enzyme form was the GFOR tetramer. The disassembly and full reactivation of GFOR from the octameric protein form was spontaneous and not dependent on exogenous NADP(H), thus precluding the dissociation of the bound nucleotide during conformational transitions and early aggregation of the enzyme. It is noteworthy that GFOR lacked the bound nucleotide after complete unfolding in 6 M GdmHCl (*20*). For the dodecameric form of GFOR, aggregation and thus inactivation was completely irreversible. The chaperonin from *E. coli*, GroEL, was previously shown to be capable of catalyzing the reversal of early aggregation steps during protein unfolding (*21*). In the case of GFOR, no evidence for GroEL-dependent disassembly of the octameric and dodecameric associates was found: compared to spontaneous enzyme reactivation in the refolding buffer lacking a denaturant, neither the yield nor the rate of renaturation of GFOR was improved when the chaperonin was present.

In contrast to in the case of GdmHCl, structural transitions of GFOR induced by urea were fully reversible. Hence, competing off-reactions leading to irreversible formation of higher aggregates were blocked efficiently in the presence of this denaturant. Functional differences between GdmHCl and urea in the unfolding of proteins have recently been pointed out and may explain these results (*22*). Urea is a monofunctional reagent that is thought to mainly affect intramolecular or intermolecular hydrophobic bonding and hydrogen bonding interactions in proteins. GdmHCl in contrast is a multifunctional reagent that is able to exert additional ionic effects on proteins. Interestingly, when an ionic reagent like SDS (0.1-0.5%, by weight) was added to GFOR in the presence of 3 M urea for reconstitution from separate components (*i.e.*, urea and SDS), the multifunctional character of GdmHCl, immediate protein aggregation was observed. It seems therefore probable that repulsive ionic interactions between individual GFOR tetramers are markedly decreased in the presence of ionic reagents, which is critical for promotion of aggregation of the enzyme.

Urea has been identified in this work as a potentially useful reagent that can prevent associate formation of GFOR and thus stabilize enzyme activity during turnover. This effect is achieved most probably through weakening of incorrect intermolecular hydrophobic and/or hydrogen bonding interactions between GFOR tetramers with a locally destabilized structure. From the results of analysis of the stability of GFOR in the presence of GdmHCl and urea one may conclude that the balance of local melting of the structure and maintenance of ionic interactions between individual tetramers is required to avoid aggregation of GFOR. In the mechanism proposed for GFOR inactivation (*12*), the deactivation of one cysteinyl residue triggers a series of conformational changes that is followed by protein aggregation. As a consequence, anti-oxidants such as dithiothreitol *together* with anti-aggregation reagents such as urea have the most pronounced effect on enzyme stability during substrate turnover. Full operational stability of GFOR for at least 24 h was observed during a fed-batch reaction in the presence of 1.0 M urea and 10 mM dithiothreitol, whereas enzyme inactivation was complete within 12 h in a control that lacked both stabilizing components.

We finally discuss the significant enzyme activation that was observed when GFOR had been inactivated by urea or GdmHCl first and then renaturated. One hypothesis that may explain this observation is a follows. The rate-determining step in the overall forward reaction of GFOR, *i.e.*, the reduction of fructose and the oxidation of glucose, is the dissociation of the glucono-δ-lactone product (*10, 11*). Hence, increasing the off-rate for the lactone is expected to result in an increase in the reaction rate at the steady state. It is well conceivable that higher intrinsic protein flexibility, at least locally, is brought about by traces of denaturant remaining in the protein structure after renaturation. This increase in conformational flexibility would then be responsible for faster dissociation of the GFOR•glucono-δ-lactone binary complex. It has been shown with other enzymes such as, *e.g.*, lactate dehydrogenase, that denaturants can increase the activity by increasing the product off-rate or the rate of decomposition of abortive enzyme•substrate complexes (*23*).

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