Dissociation and in Vitro Reconstitution of Bovine Liver Uridine Diphosphoglucose Dehydrogenase. The Paired Subunit Nature of the Enzyme†

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ABSTRACT: Uridine diphosphoglucose dehydrogenase (EC 1.1.1.22: UDPglucose dehydrogenase) at pH 5.5–7.8 is a stable homohexamer of 305 ± 7 kDa that does not undergo concentration-dependent dissociation at enzyme concentrations >5 μg/mL. Chemical cross-linking of the native enzyme at varying glutaraldehyde concentrations yields dimers, tetramers, and hexamers; at >2% (w/v) glutaraldehyde, plateau values of 21% monomers, 16% dimers, 5% tetramers, and 58% hexamers are obtained. Dissociation at acid pH (pH 2.3) or in 4–6 M guanidine hydrochloride leads to inactive monomers (M_r 52 000). Denaturation at increasing guanidine hydrochloride concentration reveals separable unfolding steps suggesting the typical domain structure of dehydrogenases holds for the present enzyme. At >4 M guanidine hydrochloride complete randomization of the polypeptide chains is observed after 10-min denaturation. Reconstitution of the native hexamer after dissociation/denaturation has been monitored by reactivation and glutaraldehyde fixation. The kinetics may be described in terms of a sequential uni–bimolecular model, governed by rate-determining folding and association steps at the monomer level. Trimeric intermediates do not appear in significant amounts. Reactivation is found to parallel hexamer formation. Structural changes during reconstitution (monitored by circular dichroism) are characterized by complex kinetics, indicating the rapid formation of "structured monomers" (with most of the native secondary structure) followed by slow "reshuffling" prior to subunit association. The final product of reconstitution is indistinguishable from the initial native enzyme.

Uridine diphosphoglucose dehydrogenase (UDP-GlcDH)[1] from bovine liver catalyzes the four-electron-transfer reaction

$$\text{UDP-Glc} + 2\text{NAD}^+ + \text{H}_2\text{O} \rightarrow \text{UDP-glucuronic acid} + 2\text{NADH} + 2\text{H}^+$$

(Feingold & Franzen, 1981). The enzyme consists of six identical subunits in octahedral assembly (Ashcom, 1985).

On the basis of present knowledge with respect to the assembly mechanism of oligomeric enzymes, the acquisition of the native quaternary structure is expected to consist of folding and association steps within a well-defined reaction sequence (Jaenicke, 1984).

In the case of UDP-GlcDH, evidence from chemical modification experiments (J. S. Franzen, personal communication) points to a paired subunit structure, suggesting that the dimer might be an important intermediate in the formation of the native hexamer. If so, this characteristic would underline the half-site activity reported for the enzyme (Franzen et al., 1976, 1978, 1980a,b, 1983).

In this paper we describe experiments concerning the dissociation and reconstitution properties of UDP-GlcDH, applying different techniques suited to monitoring the correlation of structure and function of oligomeric enzymes (Jaenicke & Rudolph, 1986). Spectral techniques and ultracentrifugation were used to explore the stability of the enzyme as well as the efficiency of reconstitution. Covalent cross-linking by glutaraldehyde during reassociation was used to achieve "snapshot" analyses of the actual particle distribution in the process of reconstitution.

Renaturation of UDP-GlcDH is a multistep process with fast recovery of spectral characteristics indicative of helix formation. The slow reconstitution of the hexamer parallels reactivation; structured monomers and dimers are the predominant intermediates on the assembly pathway.

Materials and Methods

UDP-GlcDH was prepared and assayed essentially according to the procedures of Zalitis and Feingold (1969). To remove denatured material after extended storage of the enzyme in 10 mM potassium acetate, pH 5.5, 2 mM EDTA, and 10 mM dithioerythritol at 0 °C, gel permeation chromatography on Sephacryl S-300 (column 80 cm, diameter 1.6 cm) and/or affinity chromatography with Procion green were (was) employed. Stock solutions of the enzyme (~1 mg/mL) were prepared by dialysis against appropriate buffers at ~4 °C. The specific activity of the enzyme was 3.0–3.6 units/mg (Zalitis et al., 1972). Enzyme concentration was determined spectrophotometrically by applying A_{280nm} = 1.0 cm^2·mg^{-1}; molar concentrations are based on a subunit molecular mass of 52 000 daltons.

Elution profiles from gel permeation chromatography experiments were analyzed by measuring enzymatic activity and fluorescence emission at 320 nm (λ_{ex} = 280 nm) with a Hitachi Perkin-Elmer MPF 44A spectrofluorometer. Deactivation and dissociation of the native enzyme were achieved either at pH 1.8, 0 °C, c = 0.5 mg/mL, or by 0.3–24-h incubation at 4 M

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[1] Abbreviations: Gdn·HCl, guanidine hydrochloride; $NaDodSO_4$, sodium dodecyl sulfate; UDP-GlcDH, uridine diphosphoglucose dehydrogenase (EC 1.1.1.22: UDPglucose dehydrogenase); EDTA, ethylenediaminetetraacetic acid.

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Table I: Molecular Mass of UDP-GlcDH under Native and Denaturing Conditions[a]

conditions	c_0	T (°C)	$s_{20,w}$ (S)	molecular mass (kDa) 26000 rpm	16000 rpm	9000 rpm	PAGE[b]
pH 9.0	0.1	3	12.8		312		320
pH 7.8	0.1	3	12.0	298	308	314	
pH 5.5	0.1	3	12.5	300	296	315	
pH 4.1	0.1	3	7.0, 20[c]	114	124	150–500[c]	
1 M glycine/H_3PO_4, pH 2.3	0.1	3	3.6	52	56		
0.5 M Gdn·HCl, pH 7.8	1.0	20	8.6		52–145		
1.5 M Gdn·HCl, pH 7.8	1.0	20	2.5			>500[c]	
2.0 M Gdn·HCl, pH 7.8	1.0	20	2.2		59		
4.0 M Gdn·HCl, pH 7.8	1.0	20	1.2	46	64		
6.0 M Gdn·HCl, pH 7.8	1.0	20	1.1		56		
2% $NaDodSO_4$, pH 8.0	<0.1	20					51

[a] c_0 initial concentration (mg/mL); if not stated otherwise, sodium phosphate/H_3PO_4; partial specific volume V_2 = 0.72 $cm^3\ g^{-1}$, V_2 at varying concentrations of Gdn·HCl were calculated according to Reisler and Eisenberg (1969) and Durchschlag and Jaenicke (1982), respectively. [b] Native gel electrophoresis (pH 9, 4% and 7–15% acrylamide gradient) and $NaDodSO_4$ electrophoresis. Standards: polymers of serum albumin and immunoglobulin. [c] Unspecific aggregation.

Gdn·HCl (supplied by Schwarz/Mann, Orangeburg, NY) in 0.1 M sodium phosphate buffer, pH 7.8, in the presence of 1 mM EDTA and 1–10 mM dithioerythritol (standard buffer).

For reactivation and reassociation the denaturation mixtures were diluted with standard buffer: final pH 7.8, final guanidine concentration 0.05 M. Reactivation kinetics were analyzed by taking aliquots at defined times and subsequently performing the enzyme assay [without addition of trypsin or any other quenching reagent; cf. Chan et al. (1973); Jaenicke & Rudolph (1986)]. To further characterize the enzyme, intrinsic fluorescence (Hitachi Perkin-Elmer MPF 44A) and circular dichroism (JASCO F 500A spectrophotometer, equipped with a DP-500N data processor) were used.

Sedimentation analyses were performed in a Beckman Spinco Model E ultracentrifuge equipped with a high-intensity light source and a UV scanning system ($\lambda > 235$ nm). Cells (12 mm) with sapphire windows were used in an AnG rotor. Sedimentation equilibria made use of the mensicus-depletion technique (Yphantis, 1964). To cover a wide range of enzyme concentration and to avoid artifacts due to low molecular weight impurities, at least two different scanning wavelengths and two rpm values were employed in each experiment. The partial specific volume was assumed to be 0.72 $cm^3 \cdot g^{-1}$.

Cross-linking with glutaraldehyde was done according to the procedure of Hermann et al. (1981) [cf. Jaenicke and Rudolph (1986)]. The concentration of glutaraldehyde was 2% (w/v), and the reaction time at 20 °C was 2 min. Under these conditions a plateau value of ~60% hexamers was obtained for the native enzyme; no further increase in the yield of cross-linking could be achieved at glutaraldehyde concentrations as high as 5% (w/v) (see below). Unreacted glutaraldehyde and hydrolyzable imines were reduced by adding a ~10-fold molar excess of $NaBH_4$. After 20-min incubation and addition of 5 μL of a 10 mg/mL solution of ribonuclease (as internal standard) and 20 μL of 10% sodium deoxycholate, protein was precipitated by addition of concentrated H_3PO_4. After 30-min centrifugation at 13 000 rpm (Sorvall RC 2B), the precipitate was dissolved in 50 μL of 10% $NaDodSO_4$/50 mM dithioerythritol, heated for 10 min at 100 °C, and subjected to $NaDodSO_4$–polyacrylamide gel electrophoresis (4–15% acrylamide convex gradient gels). Gel buffer: 0.38 M Tris·HCl, pH 8.8, and 0.5% $NaDodSO_4$. Reservoir buffer: 0.025 M Tris, 0.19 M glycine, and 0.1% $NaDodSO_4$. Electrophoresis was performed for 2000 V·h.

RESULTS

Equilibrium Measurements. The analysis of the reconstitution of oligomeric enzymes is based on the assumption that the molecule in its native initial state, as well as after denaturation and renaturation, represents a homogeneous system that is well-defined with respect to its physical properties. To interpret the data in terms of certain topological or kinetic properties of the native and nascent polypeptide chain, two requirements must be met: (i) the final product of reconstitution has to be indistinguishable from the initial native enzyme, and (ii) the enzyme under nondenaturing conditions must retain its state of association even at very low concentrations (Jaenicke & Rudolph, 1986).

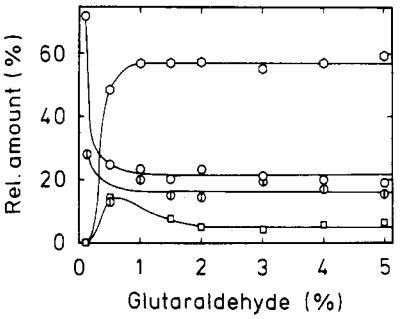

FIGURE 1: Influence of glutaraldehyde concentration on the cross-linking of native hexameric UDP-GlcDH. Covalent fixation at 20 °C by 2-min incubation in 0.1 M sodium phosphate, pH 7.8, plus 1mM EDTA at a protein concentration of 6.8 μg/mL by varying amounts of glutaraldehyde. The relative amounts of hexamers (○), tetramers (□), dimers (◐), and monomers (○) were determined by densitometry after $NaDodSO_4$–polyacrylamide gel electrophoresis.

Ultracentrifugation and spectroscopic experiments in conjunction with the assay of catalytic activity at the successive stages of denaturation/renaturation show that these two criteria are satisfied by the enzyme. Sedimentation velocity and sedimentation equilibrium experiments, and polyacrylamide gel electrophoresis (PAGE) in the absence of $NaDoSO_4$, indicate that the enzyme in its native state, i.e., at 5.5 < pH < 7.8 and 0 < T < 22 °C, is a homogeneous hexamer (Table I). There is no deviation from linearity in the ln c vs r^2 representation at enzyme concentrations beyond ~5 μg/mL. The result of cross-linking experiments appears to contradict this finding since only about 60% of the enzyme is covalently stabilized in its hexameric state. As shown in Figure 1, varying the protein:glutaraldehyde ratio does not increase the yield. At glutaraldehyde concentrations beyond 2% (w/v), all species, monomers, dimers, tetramers, and hexamers, level off to give constant values; trimers and pentamers are not detectable under the given conditions.

As has been shown by cross-linking experiments using mitochondrial malate dehydrogenase (Brückl, 1980) or

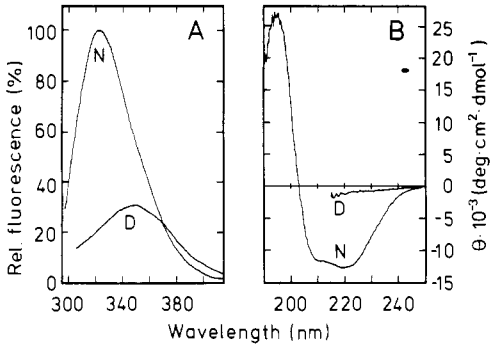

FIGURE 2: Spectral properties of native (N) and denatured (D) UDP-GlcDH. (A) Protein fluorescence, excitation wavelength 280 nm; 7.2 μg/mL protein in 0.1 M sodium phosphate, pH 7.8, containing 1 mM EDTA and 1 mM dithioerythritol; N, native enzyme; D, denatured by 4 M Gdn·HCl. (B) Far-UV circular dichroism in 0.1 M sodium phosphate, pH 7.8, containing 1 mM EDTA; N, native enzyme at a protein concentration of 1.5 mg/mL; D, denatured by 6 M Gdn·HCl; final protein concentration 73 μg/mL.

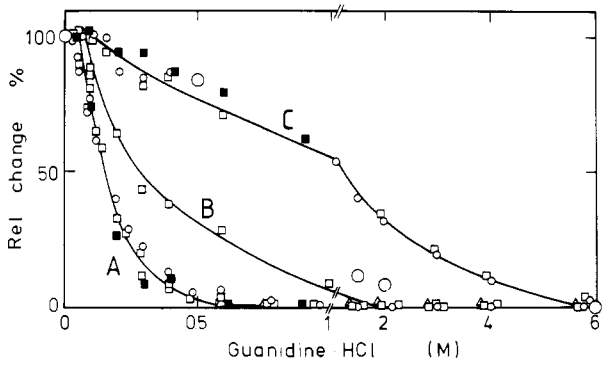

FIGURE 3: Gdn·HCl-dependent denaturation/renaturation of UDP-GlcDH as determined by (A) activity, (B) fluorescence emission at 320 nm, (C) circular dichroism at 222 nm, and (◯) sedimentation analysis ($s_{20,w}$). Experiments were performed after 10-min (○) and 24-h (□, ■) incubations at 20 °C in 0.1 M sodium phosphate, pH 7.8, containing 1 mM EDTA and 1 mM dithioerythritol, and the given amount of Gdn·HCl. Denaturation experiments (open symbols) were performed at an enzyme concentration of 9 or 37 μg/mL. Renaturation (closed symbols) after 20-min denaturation by 4 M Gdn·HCl at an enzyme concentration of 0.77 mg/mL by dilution to 6.1 μg/mL. Fluorescence data are calculated with the emission of the denatured protein taken as zero.

phosphoglycerate mutase from rabbit muscle (Hermann et al., 1983), the incomplete fixation of the native state does not necessarily indicate partial dissociation in terms of a hexamer–monomer equilibrium. It may reflect either the accessibility of lysine residues suited for bridging neighboring subunits [cf. Hajdu et al. (1972) and Friedrich et al. (1979)] or double blocking of potentially linkable interfacial lysine residues by the reagent (Ashcom, 1985).

In our work the cross-linking technique is used to compare the kinetics of regain of enzyme activity to those of the formation of intermediate oligomeric species (see below).

The fluorescence and circular dichroism spectra of the enzyme in its native and denatured states are summarized in Figure 2. A multistep transition is observed with increasing denaturant concentration (Figure 3). Loss of enzymatic activity precedes the structural changes indicated by spectroscopy. The enzyme is "fully denatured" after ≥10-min incubation only in the presence of 4–6 M Gdn·HCl.

The denaturation profiles reveal at least two separable steps in the unfolding process. Whether these steps reflect unfolding of two structural domains with significant stability differences or sequential breakdown of the tertiary and secondary structure can at present not be decided.

Table II: Comparison of Native and Reconstituted UDP-GlcDH after Denaturation in 4 M Gdn·HCl (pH 7.8) or 1 M Glycine/H_3PO_4 and Subsequent Reactivation by Dilution in 0.1 M Sodium Phosphate, pH 7.8, plus 1 mM EDTA and 1 mM Dithioerythritol, 20 °C[a]

	native	reconstituted
$s_{20,w}$ (S)	12.0 ± 0.5	12.8 ± 0.6
A (IU/mg)	3.5	3.6
λ_{max} (nm)	325 ± 1	325 ± 1
$c_{A/2}$ (M Gdn·HCl)	0.15	0.15

[a] $s_{20,w}$, sedimentation coefficient at $c_{UDP\text{-}GlcDH}$ = 0.16 mg/mL; A, specific activity; λ_{max}, maximum of fluorescence emission, λ_{ex} = 280 nm; $c_{A/2}$, midpoint of Gdn·HCl-dependent deactivation.

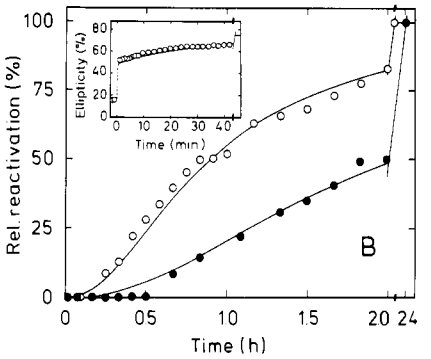

FIGURE 4: Kinetics of reactivation (renaturation) of UDP-GlcDH at 20 °C in 0.1 M sodium phosphate, pH 7.8, containing 1 mM EDTA and 1 mM dithioerythritol. Reactivation at a protein concentration of 6.8 (○) and 0.8 μg/mL (●) after 20-min denaturation in 4 M Gdn·HCl at 20 °C. Data calculated relative to a final yield of 100% (○) and 34% (●), determined after 24 h; solid line calculated for a consecutive first-order/second-order reaction with $k_1 = 4.5 \times 10^{-4}$ s^{-1} and $k_2 = 1.6 \times 10^4$ $M^{-1} \cdot s^{-1}$. (Insert) Renaturation determined under the given set of conditions by relative ellipticity at 222 nm; protein concentration was 6.5 μg/mL. Solid line calculated for a first-order reaction with $k_1 = 4.5 \times 10^{-4}$ s^{-1}.

As with all other enzymes investigated to date, reconstitution returns the enzyme to its native state [Table II; cf. Jaenicke (1984)].

Kinetic Measurements. As shown by the foregoing results, the extent of reversible denaturation of UDP-GlcDH is sufficient to justify the application of reactivation kinetics to the correlation of catalytic function with the state of association. To provide well-defined and reproducible conditions, guanidine denaturation was used in all quantitative reconstitution experiments. The variables involved in the optimization include protein concentration, residual guanidine hydrochloride, and temperature during renaturation. Under optimum conditions (UDP-GlcDH, 6 μg/mL; guanidine, ≤50 mM; temperature, 20 °C) the yield of reconstituted enzyme was in some cases close to 100%.

The recovery of enzyme activity after maximum unfolding by Gdn·HCl is governed by sigmoidal kinetics, which may be described by a consecutive first-order/second-order reaction mechanism with $k_1 = 4.5 \times 10^{-4}$ s^{-1} and $k_2 = 1.6 \times 10^4$ $M^{-1} \cdot s^{-1}$ (20 °C) (Figure 4).[2] Since reactivation kinetics monitor only the catalytically active species, quantitative analysis is possible even at low yields of reconstitution. Figure 4 shows that the rate of reactivation depends on protein concentration, proving subunit association to be required for enzymatic activity.

[2] After acid dissociation, reactivation is governed exclusively by a second-order association reaction characterized by $k_2 = 1.6 \times 10^4$ $M^{-1} \cdot s^{-1}$ at 20 °C. The difference in the reaction order of the rate-determining steps comparing reactivation from acid dissociation and from guanidine dissociation suggests that the former process leaves some backbone structure of UDP-GlcDH preserved, as with other dehydrogenases (Jaenicke & Rudolph, 1983).

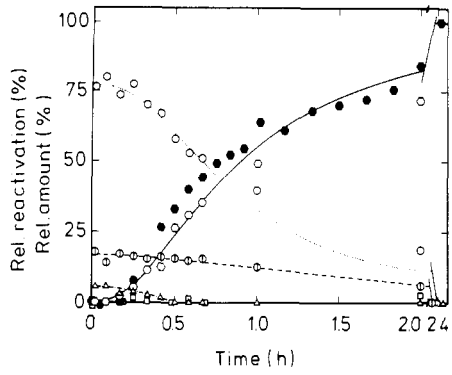

FIGURE 5: Kinetics of reassociation and reactivation of UDP-GlcDH after denaturation by 4 M Gdn·HCl as described in the caption of Figure 4. Reassociation by snapshot analysis with glutaraldehyde fixation and subsequent $NaDodSO_4$ gel electrophoresis at a protein concentration of 6.8 μg/mL. Relative amounts of hexamers (○), tetramers (□), trimers (△), dimers (◐), and monomers (○) are corrected for incomplete fixation of native hexamers (cf. Figure 1). Reactivation (●) calculated relative to a final yield of 100%, as determined after 24-h renaturation. The solid line is calculated for a consecutive first-order/second-order reaction with $k_1 = 4.5 \times 10^{-4}$ s^{-1} and $k_2 = 1.6 \times 10^4$ $M^{-1} \cdot s^{-1}$.

Analysis of the *folding* process by monitoring the regain of native dichroic absorption at 222 nm yields biphasic kinetics with a very rapid major amplitude reflecting fast formation of secondary structure, followed by a minor slow phase that may be attributed to tertiary structure formation (Figure 4 insert).

An additional increase in ellipticity during quaternary structure formation eventually escapes detection due to the limited sensitivity and poor long-term stability associated with the circular dichroic measurements.

The *association* kinetics were determined by cross-linking with glutaraldehyde. With the application of optimum conditions (2-min incubation with 2% (w/v) glutaraldehyde at 20 °C), it is evident that monomers are transformed into hexamers via dimeric intermediates, while trimers do not exceed trace amounts (Figure 5). To a first approximation, the increase in native hexamer corresponds to the decrease in the relative amount of the monomer, reactivation strictly parallels hexamer formation, and intermediates do not exhibit appreciable activity (Figure 5).

Discussion

Uridine diphosphoglucose dehydrogenase is a homohexamer that exhibits half-site activity (Feingold & Franzen, 1981; Franzen et al., 1980a,b, 1983). From electron microscopy and chemical cross-linking studies, the conclusion has been drawn that the six subunits are organized in space about an octahedral frame (Ashcom, 1985). Hence the enzyme could be alternatively considered as a trimer of dimers or a dimer of trimers.

The present reconstitution experiments shed more light on the topology of the enzyme. Dissociation, denaturation, and inactivation of the enzyme were accomplished under a variety of solvent conditions (Table I). In general, dissociation was accompanied by partial denaturation and by complete loss of enzyme activity, suggesting that only hexamers are catalytically active.

Increasing concentrations of Gdn·HCl cause a complex denaturation behavior (Figure 3). Complete loss of enzyme activity occurs at guanidine concentrations as low as 0.6 M (midpoint of transition, 0.15 M). On the other hand, structural transitions occur in a multiphasic fashion. The fluorescence emission profile reflects separable transitions at 0.15 and 0.7 M, while circular dichroism (θ_{222}) indicates an additional broad transition that finally leads to the fully randomized polypeptide chain at ~4 M Gdn·HCl. The structural transitions are reversible without significant hysteresis.

Obviously the enzyme in its native and renatured states is indistinguishable (Table II). This is of critical importance in employing reconstitution experiments to elucidate the structure–function relationship of an enzyme.

In order to optimize the extent of reconstitution, a number of variables have to be considered (Jaenicke & Rudolph, 1986). To minimize competing aggregation reactions (Jaenicke, 1974; Zettlmeissl et al., 1979), the concentration of the enzyme must not exceed an upper limit of 40 μg/mL. As reported previously, starting renaturation from the fully denatured state provides optimum reconstitution conditions.

Attempts to resolve the mechanism of quaternary structure formation of oligomeric enzymes led to a general kinetic scheme, comprising consecutive unimolecular folding and bimolecular association reactions, with dimerization steps at the monomer or dimer level being the essential rate-limiting processes (Jaenicke, 1984).

By application of this scheme to the present system, dimeric intermediates in the assembly pathway would suggest a paired subunit arrangement in the sense that the final product represents some kind of dimer assembly. To obtain independent experimental evidence for the involvement of dimers in the assembly process, reactivation kinetics and cross-linking were used.

Kinetic analysis of reactivation and renaturation has been widely used as a tool in the analysis of the correlation of folding, association, and enzymatic function. The rationale is that during renaturation, denatured subunits must fold to form "structured monomers," which can then recognize each other to form the native oligomer. The kinetics of restoration of enzyme activity may be used to identify the minimum catalytic entity. Thus, if the monomer exhibits full enzymatic activity, reactivation must be independent of the state of association; i.e., it must obey first-order kinetics. If, on the other hand, association steps are rate-determining (at least in a certain range of enzyme concentration), the subunit cannot be fully active.

The correlation between restoration of enzyme activity and the appearance of a particular oligomer can be deduced by using bifunctional (cross-linking) reagents to fix the particle distribution corresponding to a given time of reassociation. In order to apply this "time-resolved population analysis", the cross-linking procedure has to fulfill three requirements: it should be *complete* within a reasonably *short time* (short compared to the reassociation time), and it should *not interfere* with the mechanism of reconstitution. Glutaraldehyde has been found to be the most versatile reagent fulfilling these conditions (Hermann et al., 1979, 1981).

In the present case, optimum reconstitution conditions leading to a sufficiently high yield of reconstitution did not reach more than ~60% cross-linking (Figure 1). However, since the kinetic analysis requires *relative* amounts and *relative* reactivation only (referring to the final yield as 100%), the incomplete cross-linking reaction does not necessarily interfere with the mechanistic interpretation of the data.

Considering the relative amounts of the various oligomeric species, from the monomer to the hexamer, at varying glutaraldehyde concentrations upon cross-linking of the native enzyme, plateau values are obtained at >2% (w/v) glutaraldehyde (Figure 1).

It is significant that the total population does not contain detectable amounts of trimers or pentamers; apart from the

hexamer, only oligomers with even numbers of subunits are present. This holds for the *equilibrium* situation, which should reflect the topology of the oligomer with respect to the nearest-neighbor relationships of its constituent subunits (Figure 1). In connection with the *kinetics* of reconstitution where glutaraldehyde fixation is used as a "snapshot technique" to trap intermediates, again the trimer occurs only in trace amounts (Figure 5); the pathway of the monomer → hexamer transition involves dimer almost exclusively [apart from monomeric intermediates (see below)]. This is in accordance with the paired character of the subunit arrangement in the native quaternary structure, which may be correlated to the half-site reactivity of the enzyme (Franzen et al., 1980a,b, 1983).

The decrease in monomer concentration during reconstitution of the enzyme deviates markedly from normal exponential behavior. Slow folding at the monomer level is involved as a rate-determining step before dimerization takes place (Figure 5).

At least two phases in the regain of native circular dichroic absorption can be distinguished during reconstitution. A fast reaction [which has been frequently observed in the renaturation of oligomeric enzymes, e.g., Jaenicke and Rudolph (1983); Hermann et al., 1983] may be attributed to the recovery of most of the secondary structure. Subsequently, the structural elements coalesce to form the "structured monomer", which then undergoes association (Figure 4 insert).

As in the case of a variety of oligomeric enzymes investigated previously, the kinetic scheme describing the given kinetic behavior would comprise as a minimum two rate-limiting processes

$$6\mathcal{M} \xrightarrow{\text{fast}} 6M' \xrightarrow{\text{slow}} 6M \xrightarrow{\text{slow}} 3D \xrightarrow{\text{fast}} D + T \xrightarrow{\text{fast}} H$$

where $\mathcal{M}$, M′, and M represent the monomer in its unfolded, partially folded, and "structured" form, respectively, D represents the "structured dimer", T tetramers, and H the native hexamer.

In the cross-linking pattern, the monomer population comprises M′ and M. The concentration dependence of reactivation (Figure 4) and the synchrony of reactivation and hexamer formation (Figure 5) suggest that both M′ and M are enzymatically inactive and that the hexamer is the only active species.

The sigmoidicity of the reactivation profiles (Figures 4 and 5) reflects the slow formation of "structured monomers" (M) when reconstitution starts from the fully unfolded polypeptide chains. With less drastic denaturation conditions, e.g., short incubation at pH ~2, reconstitution seems to set out from M, as indicated by the simple second-order reaction kinetics observed after acid denaturation.

The necessity of involving a dimeric intermediate to account for the kinetics of reassembly of UDP-GlcDH is compatible with the octahedral arrangement of the enzyme subunits and with the trimer of dimers nature of this arrangement as inferred from electron microscopy and chemical modification (Hill, 1984; Ashcom, 1985).

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Registry No. UDP-GlcDH, 9028-26-6; Gdn·HCl, 50-01-1; glutaraldehyde, 111-30-8.

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