Denaturation and Renaturation of Bovine Liver Glutamic Dehydrogenase after Dissociation in Various Denaturants

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Oligomeric glutamic dehydrogenase from bovine liver is dissociated to inactive monomers ($M_r = 56\,000$) under a wide variety of conditions: $3 \geq pH \geq 12$, 6 M guanidine · HCl, 6 M urea, 0.2% sodium dodecylsulfate. High hydrostatic pressure (< 1 kbar) only affects the association equilibrium of the native hexamer to higher polymers. The respective reaction volume ($\Delta V = 28 \pm 5$ ml · mol^{-1} at 298 K, 1 bar) is linearly dependent on temperature and pressure. At $p > 1.5$ kbar dissociation of the hexamer occurs; this reaction is accompanied by irreversible deactivation.

Depending on the denaturant applied for the monomerization, the final conformational state of the polypeptide chain differs widely regarding its residual structure.

As taken from laser light scattering measurements the rate of dissociation at pH 1.8 follows first order kinetics with a rate constant $k_1 = 0.42 \pm 0.06$ s^{-1}.

In the range of the oligomer $\rightleftharpoons$ monomer transition, dissociation is accompanied by irreversible aggregation leading to inactive high molecular weight material. At low concentration ($c < 5\,\mu g/ml$) this side reaction can be slowed down, so that the reconstitution of the enzyme can be monitored using spectroscopic techniques. Concentration dependent stopped-flow experiments prove the regain of fluorescence to be a rapid first order process; the respective half-times at pH 7.4 are $\tau_{1/2} = 2.0 \pm 0.5$ ms and 0.7 ± 0.2 ms for the "renaturation" from 6 M guanidine · HCl, pH 6, and pH ~ 2, respectively.

The product of reconstitution shows the fluorescence and circular dichroism pattern characteristic for the native enzyme. However, no reactivation can be achieved under any of the following conditions: optimum protection against chemical modification; variation of enzyme concentration, temperature, and hydrostatic pressure; addition of specific ligands such as coenzymes, substrates, ADP, membrane constituents (cardiolipin, electron transfer particles ETPH). Obviously, the "renaturation" (D → N) of glutamic dehydrogenase is governed by a side reaction according to

$$N \rightarrow D \rightleftharpoons R \rightarrow A$$

which causes aggregation of intermediates R instead of reconstitution of the native enzyme.

Introduction

Glutamic dehydrogenase from bovine liver is a hexameric enzyme consisting of identical subunits. Above a critical concentration the hexamers underly a reversible polymerization-depolymerizaton equilibrium which may be quantitatively described by an isodesmic model, depending on solvent conditions, temperature, pressure, and specific effectors as parameters [1, 2]. Aside from one preliminary report [3] there has been no experimental evidence in the past indicating that glutamic dehydrogenase may be reconstituted after denaturation and dissociation. Recent experiments seem to indicate that the enzyme cannot be renatured [4]. Since oligomeric enzymes have in general been found to be accessible to reconstitution [5] this result is striking and provides a challenge to either find conditions to reactivate the enzyme, or to characterize the products formed upon reestablishing quasi-physiological solvent conditions.

The following experiments refer

(i) to the exploration of conditions allowing to reconstitute the enzyme,
(ii) to the kinetic analysis of the partial regain of native fluorescence upon "renaturation", and
(iii) to the physico-chemical characterization of the enzyme in its native, denatured, and "renatured" states.

Abbreviations: ETPH, electron transfer particles; N, D_i, R, native, denatured and renatured states of the enzyme; SDS, sodium dodecyl sulfate.

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Materials and Methods

Glutamic dehydrogenase from bovine liver (EC. 1.4.1.3), NAD^+, NADH, ATP, ADP were purchased from Boehringer, Mannheim; guanidine · HCl and

urea (ultrapure) from Schwarz/Mann, New York; dithioerythritol from Roth, Karlsruhe; bovine serumalbumin, cardiolipin, SDS and acrylamide from Serva, Heidelberg. All other reagents were of A-grade purity: Merck, Darmstadt.

Quartz bi-distilled water was used throughout.

Homogeneous solutions of cardiolipin were prepared by sonication according to Fleischer and Fleischer [6], submitochondrial particles (ETPH) according to Myers and Slater [7], and Hansen and Smith [8].

Stock solutions of the enzyme were obtained by dialyzing the original ammonium sulfate suspension or glycerol solution of the enzyme against standard buffer (0.2 M potassium phosphate, pH 7.6, 1 mM EDTA, 2.5 mM dithioerythritol) at 4 °C and subsequent millipore filtration (0.8 μm). Final concentration after dialysis 4–16 mg/ml. Enzyme concentrations were calculated from $A_{279}^{0.1\%} = 0.97\ cm^2 \cdot mg^{-1}$ [9]. The specific activity was 45–60 IU/mg: enzyme assay at 366 nm (25.0 °C) in 0.17 M potassium phosphate pH 7.6, 14 mM α-ketoglutarate, 0.22 M ammonium acetate, 0.13 mM NADH, 1.7 mM ADP, plus 1.5 nM glutamic dehydrogenase. Storage of the stock solution of the enzyme at 4 °C under nitrogen leads to a constant specific activity over several weeks. Protein determinations for the ETPH preparations made use of the biuret method [10].

For deactivation (reactivation) experiments the stock solution (~ 5 mg/ml) was diluted 10 fold (≧ 200 fold) with the respective deactivation (reactivation) buffers.

To measure the kinetics of reconstitution the specific activity was monitored during the first three hours after dilution, and after 24 h. The activity of the untreated native enzyme under reactivation conditions was used as a reference.

Absorption and fluorescence spectra were measured in Cary (118-c) and Hitachi-Perkin-Elmer (MPF 44 A) spectrographs. Circular dichroism spectra were recorded on a Roussel-Jouan Dichrographe II.

Stopped flow experiments made use of a Durrum-Gibson Spectrophotometer equipped with a fluorescence attachment, Tectronix oscilloscope 6723 A, and Datalab DL 905 transcient recorder (Canberra). High pressure turbidity measurements were performed in a 12 mm transmission cell at $\lambda = 320$ nm and $p = 1-2000$ bar; for experimental details *cf.* [11]. Calculations of the dissociation volumes were performed as described in refs [12, 13].

Sodium dodecyl sulfate-polyacrylamide gel electrophoresis was performed according to Maizel [14]. Ultracentrifugal analyses were performed in a Beckman Model E analytical ultracentrifuge.

Results and Discussion

Experimental results characterizing the enzyme in its native (N), denatured (D_i) and "renatured" states (R) are summarized in Table I and in Fig. 1. As frequently observed, the subunits produced by acid treatment (pH 2.3), or denaturation in 6 M guanidine · hydrochloride differ considerably in their residual structure. In 6 M guanidine · hydrochloride fully denatured monomers are obtained while acid treatment leads to partially structured but slowly aggregating entities [15]. Upon renaturation under standard conditions, a strong tendency of the refolding subunits to from "wrong aggregates" is observed.

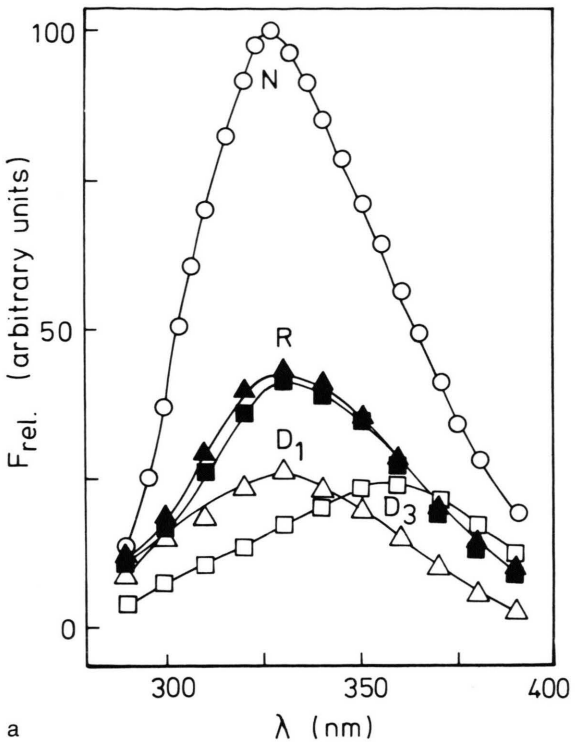
Table I. Characterization of glutamic dehydrogenase in its native, denatured and "renatured" states [a].

	N 7.6 ≧ pH ≧ 5.0	D_1 [b] pH 2.0	D_2 pH 12.0	D_3 6 M Guanidine · HCl	R [c] pH 7.6
spec. activity [%]	100	0	0	0	0
$s_{20,w}$ [S]	13.0 ± 0.6	2.2 ± 0.4	2.2 ± 0.4	1.3 ± 0.2	
λ_{max} (fluorescence) [nm]	328 ± 1	330 ± 4		352 ± 4	330 ± 4
rel. fluorescence [%]	100	27 ± 2		26 ± 2	42 ± 3
$-\Theta_{222}$ [degree · cm² · dmol⁻¹]	12 700 ± 500	5 200 ± 500		2 200 ± 300	8 400 ± 700

[a] N, D_i, R = native, denatured, and "renatured" states, respectively. 0.1 M potassium phosphate buffer. Sedimentation analysis at $c = 0.1$ mg/ml; spectroscopic measurements at $c = 5$ μg/ml.

[b] As taken from laser light scattering measurements (*cf.* [13, 17]), the rate of dissociation ($N \rightarrow D_1$) at pH 1.85 follows first order kinetics with a first order rate constant $k_1 = 0.42 \pm 0.06\ s^{-1}$ (20°).

[c] Heterogeneous distribution. Spectroscopic measurements performed 0.3–30 min after readjusting the solvent to quasi-physiological conditions. Within this time range, aggregation does not affect the spectra significantly; after 24 h complete aggregation occurs, so that low speed centrifugation (20 min, 48 000 × *g*) is sufficient to spin down the enzyme.



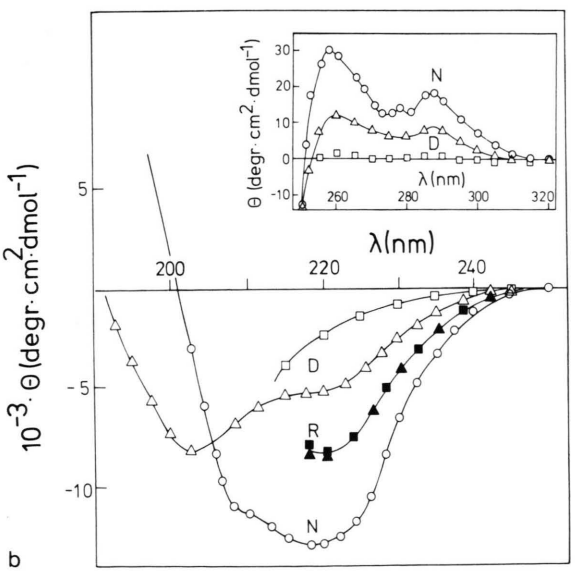


Fig. 1. a) Fluorescence emission spectra of native (N), denatured (D), and "renatured" (R) glutamic dehydrogenase. $\lambda_{exc} = 280$ nm, enzyme concentration $c = 5$ µg/ml, 1 mM EDTA, 5 mM dithioerythritol, 20 °C. (○) N in 0.1 M potassium phosphate buffer pH 7.6; (△) D_1 in 0.1 M potassium phosphate buffer pH 2.0; (□) D_3 in 6 M guanidine · HCl pH 6.0; (▲, ■) R in 0.1 M potassium phosphate buffer pH 7.6, after denaturation at pH 2.0 (▲) and 6 M guanidine (■), respectively. b) Circular dichroism spectra of native (N), denatured (D), and renatured (R) glutamic dehydrogenase. Enzyme concentration $c = 0.1$ mg/ml (far-UV region) and 1.0 mg/ml (near-UV region, insert); oxygen-free, 0.1 mM EDTA, 0.1 mM dithioerythritol, 21 °C. Symbols as in Fig. 1 a.

Obviously, a side reaction competes with the process of reconstitution [16, 17]. Applying low enzyme concentrations ($c \sim 5$ µg/ml ≙ 90 nM), aggregation may be kept sufficiently slow to allow the spectroscopic characterization of the reconstituting intermediary product of renaturation (R). As documented in Table I, R represents the inactive enzyme fixed in a partially renatured state.

Concentration dependent stopped flow experiments prove that the regain of fluorescence in the reaction $D_i \rightarrow R$ is a rapid first order process. The respective half-times are $\tau_{1/2} = 2.0 \pm 0.5$ ms and $\tau_{1/2} = 0.7 \pm 0.2$ ms for the renaturation from 6 M guanidine · hydrochloride and pH 2.3, respectively. This finding indicates that the fluorescence change monitors a transconformation rather than an association reaction. The previously mentioned failure to recover the native conformation of glutamic dehydrogenase using conventional denaturation-renaturation techniques led us to systematically search for dissociation conditions where reversible deactivation might be achieved. Methods applied were (i) optimum protection against chemical modification, (ii) variation of enzyme concentration and temperature, (iii) elevated hydrostatic pressure [11, 12], (iv) guanidine · hydrochloride or urea at neutral and acid pH (for optimum conditions *cf.* [18]), (v) partial deactivation in the transition range of dissociation, and finally (vi) potential specific effectors, like coenzymes, substrates, ADP, and membrane constituents or vesicles (Table II).

(i) Previous experiments have shown that protecting agents, like glycerol, saccharose, serumalbumin, as well as the application of oxygen-free buffers may be used to optimize the yield of reconstitution. Changing the concentrations of the given additives (≦ 50 vol% glycerol; ≦ 1 M saccharose; ≦ 10 mg/ml BSA) does not provide measurable reactivation.

(ii) If unspecific aggregation and reactivation show significant differences in their respective activation energies, the ratio of their rate constants is expected to depend significantly on temperature. Attempts to reactivate the enzyme at 0 and 40 °C

Table II. Conditions of denaturation and "renaturation" [a].

Denaturation		Renaturation
$3 \leqq pH \leqq 12$	0.1 – 0.2 M KP	(i) *Dialysis* against 0.2 M KP pH 7.6
6 M Gdn · HCl	0.1 M KP pH 7.6	(ii) *Dilution:*
6 M Gdn · HCl	0.1 M NaC pH 2	with oxygen-free buffer solutions, (reconstitution under nitrogen);
6 M urea	0.1 M KP pH 7.6	to very low enzyme concentration;
0.2% SDS	0.1 M KP pH 7.6	in the presence of stabilizing agents:
1.5 – 2.0 kbar	–	glycerol ($c \leqq 50$ vol%),
pH 3.8 – 4.5	0.1 – 0.2 M KP [b]	saccharose ($c \leqq 1$ M),
		serum albumin ($c \leqq 10$ mg/ml);
		at varying temperature (0–40 °C);
		in the presence of lipids (cardiolipin), or membrane vesicles (ETPH);
		(iii) *Pressure release* (~ 2 kbar $\rightarrow$ 1 bar)

[a] KP, potassium phosphate; NaC, sodium citrate, 1 – 5 mM EDTA, and 1 – 10 mM dithioerythritol were added to all buffer solutions; Gdn · HCl, guanidine · hydrochloride.
[b] partial deactivation in the transition range of acid induced deactivation.

failed, although the native enzyme is found to be fully active in the given temperature range for at least 24 h.

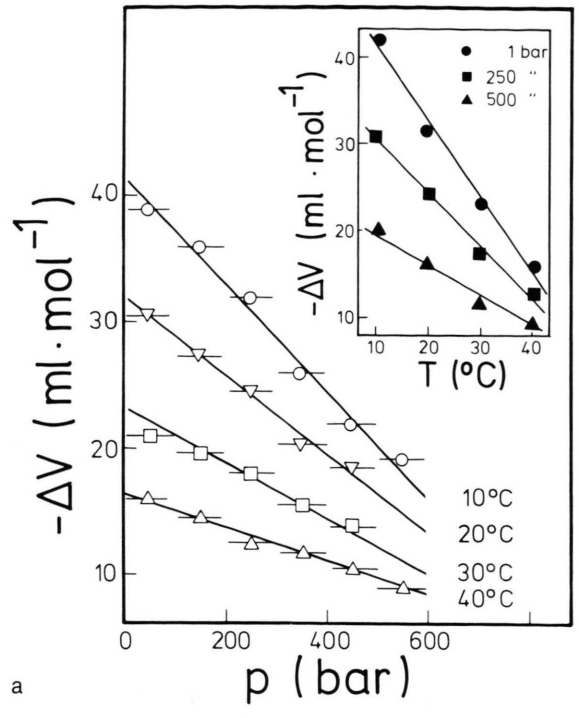
(iii) As demonstrated for LDH [11, 19], high hydrostatic pressure ($p = 1-2$ kbar) is the only parameter which has been shown to cause fully reversible deactivation and dissociation. In the present case, systematic changes of temperature and pressure indicate a complex dependence of the native polymeric structure of the enzyme on pressure. A reversible shift of the dissociation equilibrium of the hexamer

$$n\,M_6 \rightleftharpoons (M_6)_n \qquad (1)$$

is observed at $p \leqq 1$ kbar and $T \leqq 40$ °C (0.2 M Tris/HCl or 0.2 M phosphate buffer pH 7.6; 0.1 mM EDTA, 0.1 mM dithioerythritol; saturated with nitrogen); at higher pressures and temperatures irreversible aggregation occurs. Dissociation of the native oligomer (M_6) cannot be detected under the given conditions (*cf.* [12]). As shown in Fig. 2, there is a linear dependence of the reaction volume of polymerization ΔV on p and T. The respective linear relation of ΔV and the thermodynamic parameters of the polymerization reaction ΔH^0 and ΔS^0 suggests the solvent to be involved in the dissociation reaction of the polymeric species $(M_6)_n$. Applying $p \sim 2$ kbar (20 min) in 0.1 M Tris/HCl leads to complete deactivation, while phosphate under identical conditions protects the enzyme from deactivation and dissociation. This is in agreement with the dissociation behaviour of lactic dehydrogenase [11]; it corroborates the stabilizing effect of multivalent ions discussed previously by von Hippel and Wong [21]. The pressure induced deactivation in Tris/HCl turns out to be irreversible (in contrast to the results obtained with a number of other oligomeric enzymes (Müller, Seifert, Jaenicke, unpublished)). No regain of enzymatic activity was achieved in the case of glutamic dehydrogenase.

(iv) Applying strong denaturants (which have been shown to provide maximum reconstitution yields for a number of enzymes [16, 18]), no reactivation has been accomplished in 6 M urea (0.1 M phosphate pH 7.6) or 6 M guanidine · hydrochloride 0.1 M phosphate pH 7.6 and 0.1 M citrate pH 2.3) at enzyme concentrations $c = 0.4-25$ µg/ml.

(v) The same holds if reactivation is attempted in the transition range of the pH dependent deactivation at $3.8 \leqq pH \leqq 4.5$, corresponding to 40–90% residual activity, or in the presence of low guanidine · hydrochloride concentrations which are known to increase the flexibility of the polypeptide chains combined in the native oligomer (*cf.* [22]). As shown in Fig. 3, deactivation in the given range is paralleled by denaturation, dissociation, and subsequent aggregation. To reduce the latter effect, enzyme concentrations as low as 8 nM were applied in the experiments. Changes of the incubation time (2–7 h), or the reactivation buffer (pH 6.7–8.7) did not provide any detectable increase of the residual activity within the limits of error (Table III). Similar experiments using the enzyme partially deactivated by guanidine · hydrochloride (2.7 M), urea (6 M), or high pressure (1.5 kbar) led to the same (negative) result.



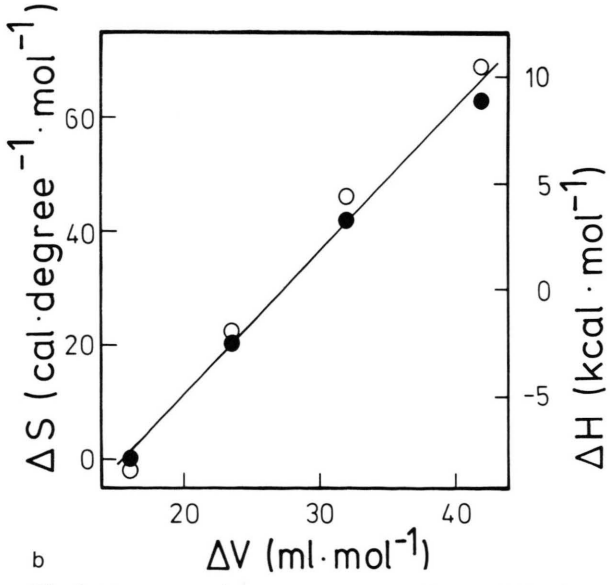


Fig. 2. Pressure and temperature dependence of the reaction volume of polymerization-depolymerization of glutamic dehydrogenase. Enzyme concentration $c = 3.0$ to 3.4 mg/ml, 0.2 M potassium phosphate pH 7.6, 0.1 mM EDTA, 0.1 mM dithioerythritol. a) Reaction volume of depolymerization as a function of pressure. Insert: Reaction volume of depolymerization as a function of temperature. b) Dependence of the reaction volume (ΔV) of the polymerization equilibrium (Eqn (1)) on ΔH (○) and ΔS (●) at 25 °C and 1 bar. ΔH and ΔS data taken from ref. [20].

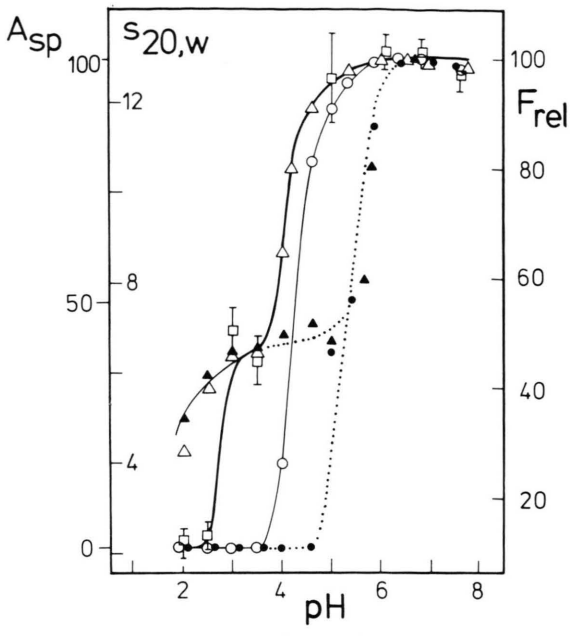


Fig. 3. pH dependent dissociation, denaturation, and deactivation of glutamic dehydrogenase in the acid pH range. 0.1 M potassium phosphate buffer, plus 1 mM EDTA and 10 mM dithioerythritol, ~ 20 °C. Full lines: 3–4 h incubation; dotted lines: 144 h incubation. *Dissociation*, measured by sedimentation velocity (□). $c = 0.1$ mg/ml, 12 mm double sector cells, 40000 and 60000 rpm; duration of sedimentation 3 h. at pH 4.5 ± 0.5 fast aggregation of the enzyme occurs. *Denaturation*, monitored by fluorescence emission at 330 nm ($\lambda_{exc} = 280$ nm) (△, ▲); $c = 25$ µg/ml. *Deactivation* at $c = 25$ µg/ml (○, ●).

(vi) The fact that glutamic dehydrogenase is found to be inaccessible towards reactivation may be caused by the lack of essential "effectors" or ligands in the reconstitution *in vitro*, compared to the situation *in vivo*. Possible canditates might be cofactors known to form binary complexes with the enzyme; on the other hand, membrane components need consideration, since there is some evidence that glutamic dehydrogenase interacts with the inner mitochondrial membrane.

In the present reconstitution experiments, the effect of coenzymes (≦ 15 mM NADH of NAD^+); substrates (≦ 50 mM α-ketoglutarate, or glutamic acid); ADP (≦ 5 mM); cardiolipin (2.4–240 µM), and submitochondrial particles (ETPH, ~ 10–165 µg/ml) was investigated.

None of these additives has been found to initiate reconstitution; cardiolipin shows an inhibitory effect on the enzyme; the same holds for the ETPH, in

Table III. *Renaturation experiments with glutamic dehydrogenase* partially deactivated at pH 3.8 – 4.5. Deactivation at $c = 92$ µg/ml ≙ 1.6 µM in 0.1 M potassium phosphate buffer in the presence of 1 mM EDTA and 5 mM dithioerythritol at 20 °C. Native GluDH: $A_{sp} = 45$ IU/mg. Reactivation after up to 48 h incubation at given pH and final concentration (c). Error limits give standard deviations of 4 – 8 measurements.

Deactivation at		Residual activity	"Reactivation" (IU/mg) at			
pH	incubation time [h]	[IU/mg]	c (nM)	pH 6.7	pH 7.6	pH 8.7
4.50	4.5	39.6 ± 1.6	8.2	38.7 ± 1.5	41.4 ± 1.4	40.4 ± 1.5
			82.0	41.3 ± 1.7	42.8 ± 2.0	39.5 ± 1.4
4.20	4.0	35.4 ± 1.4	8.2	38.5 ± 0.7	38.2 ± 0.7	39.2 ± 1.8
			82.0			37.6 ± 0.9
4.15	4.25	26.0 ± 1.4	8.2		24.2 ± 0.9	
			82.0		24.4 ± 1.0	
			164.0		24.9 ± 0.9	
4.10	6.5	19.9 ± 0.6	8.2		19.2 ± 0.8	
			16.4		18.5 ± 0.7	
			32.8		19.0 ± 0.7	
			82.0		19.9 ± 0.6	
			164.0		20.9 ± 0.8	
3.80	2.0	18.0 ± 0.5	16.4		19.6 ± 0.7	
			82.0		17.7 ± 0.3	

spite of their weak intrinsic glutamic dehydrogenase activity. Under standard test conditions the enzyme is not affected by the two membrane components, as demonstrated by control tests with native glutamic dehydrogenase.

Conclusions

The abovementioned experimental results prove that GluDH (contrary to other NAD dependent dehydrogenases) cannot be reversibly dissociated and deactivated, applying a wide variety of conditions. Since previous attempts in the same direction were also unsuccessful ([4], P. C. Engel, C. Frieden, H. Sund, C. Veeger, personal communications) we tried to characterize the various denatured states (D_i) and the "renatured" state (R) in order to compare them to the enzyme in its native state (N). As taken from the spectroscopic data, the extent of denaturation in the various denaturants differs widely. Under all conditions the formation of "wrong aggregates" (A) according to

$$N \rightleftharpoons D_i \rightarrow A \qquad (2)$$

(*cf.* [17]) severely disturbs renaturation. At low enzyme concentration (~ 5 µg/ml) the irreversible side reaction can be slowed down considerably, so that intermediates of renaturation become accessible upon reestablishing quasi-physiological solvent conditions. As indicated by spectroscopic evidence (Table I, Fig. 1 a), the fluorescence emission and far-UV circular dichroism of the renatured system resembles the native enzyme. However, the regain of native-like optical properties is not accompanied by reactivation.

Obviously, the partially structured monomeric state (which is indicated *e. g.* by the concentration-independent fast regain of fluorescence) does not provide the correct subunit interfaces required to generate the native hexamer.

The following reaction scheme is suggested to describe the observed denaturation-renaturation pathway

$$N \rightarrow D_i \rightleftharpoons R \rightarrow A. \qquad (3)$$

In this scheme, $D_i \rightleftharpoons R$ does not represent a true denaturation-renaturation equilibrium, because N, R, and A refer to quasi-physiological conditions, while D_i stands for the enzyme in the presence of the various denaturants. The fact that R can be converted back to D_i has been established by extensive studies using a number of enzymes [17].

The given result which is corroborated by recent renaturation experiments by Sugrobova *et al.* [4] suggests that in the case of glutamic dehydrogenase the vectorial synthesis or some kind of post-translational processing may be of importance in the process of folding. Since membranes or membrane components might be expected to have a "nucleating effect" on the reconstitution process, cardiolipin and ETPH were added to the reconstitution medium. Inhibition of enzyme activity proves both to interact with the enzyme [23, 24]; however, no positive effect on the recovery of enzymatic activity could be detected.

The alternative hypothesis that proteolytic modification ("nicking") of the native polypeptide chain is responsible for the unsuccessful attempts to reconstitute the enzyme, is disproved by SDS-polyacrylamide gel-electrophoresis which shows only *one* band with the correct subunit molecular weight [13]. Of course, the loss of minor terminal sequences may escape detection in this approach [25].

Aside from the previously mentioned extrinsic effects such as "nucleation" or chemical modification, intrinsic properties of the polypeptide chain may block the reconstitution process. The hexameric quaternary structure, as well as the anomalous association behaviour indicate that glutamic dehydrogenase shows special features compared with other NAD-dependent dehydrogenases investigated so far. If processing of the nascent chain determines these features, cloning of the (hypothetical) pro-enzyme should be attempted.

Acknowledgements

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