

Reversible pH-Induced Dissociation of Glucose Dehydrogenase from *Bacillus megaterium*

II. Kinetics and Mechanism

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Glucose Dehydrogenase, Subunit Dissociation, *Bacillus megaterium*

Glucose dehydrogenase from *Bacillus megaterium* exists as a stable, active tetramer at pH 6.5. By shifting the pH to 9, the enzyme is, completely and reversibly, dissociated into four inactive protomers. Kinetics and mechanism of this pH-induced dissociation have been studied, at various enzyme concentrations, by ultraviolet absorption, circular dichroism, normal and stopped-flow fluorescence as well as by light scattering and activity measurements. Dissociation of the fully active tetramer proceeds via three distinct kinetic steps: (1) fast conformational rearrangement of the tetramer, without any loss of activity ($t_{1/2}$ 0.0075 sec); (2) slow isomerization to a tetramer with lower specific activity ($t_{1/2}$ 27 sec); (3) subsequent dissociation of this rearranged tetramer into inactive monomers ($t_{1/2}$ 114 sec) with still intact native secondary structure. All three processes follow first-order kinetics. Both rate and extent of the dissociation are reduced, with a concomitant shift to higher reaction orders, by increasing the NaCl concentration in the buffer. This suggests the establishment of a dissociation/association equilibrium, due to the concentration-dependent stabilization of the tetrameric enzyme state by NaCl.

Introduction

In a preceding communication [1], we have reported our investigations by various spectroscopic methods of the changes in structure and functional properties of glucose dehydrogenase from *Bacillus megaterium* upon alkaline dissociation (β -D-glucose: NAD(P)⁺ 1-oxido-reductase, EC 1.1.1.47). Circular dichroism, ultra-violet and fluorescence measurements demonstrate that intrinsic tryptophan chromophores from the protein core or the subunit contact region, respectively, are exposed to the solvent medium upon dissociation. Comparison with the results from light scattering measurements revealed tryptophan residues, as well as the enzymatic deactivation, not to be strictly concomitant with quaternary structure degradation. We have interpreted this discrepancy in terms of a slight rearrangement within the protein structure, coupled with a reduction in specific activity, preceding the actual dissociation step. Circular dichroism backbone spectra displayed virtually no changes in secondary structure between the native tetramer and the monomeric state of glu-

cose dehydrogenase. The dissociation process was thus established to be restricted simply to the dissolution of the quaternary structure, with only slight changes in tertiary structure. All structural and functional changes which occur in the course of the dissociation are fully reversible by simply readjusting the pH to 6.5.

The mild dissociation conditions (pH 8–9), the absolute reversibility of all structural and functional changes, and the preservation of secondary structure upon dissociation are rather unique properties of glucose dehydrogenase, compared with other dissociating enzyme systems. With very few exceptions [2, 3], dissociation of oligomeric enzymes to our knowledge is always accompanied by drastic and either partially or totally irreversible conformational changes [4–9]. The lack of such irreversible processes makes glucose dehydrogenase the ideal model for a kinetic investigation of the dissociation mechanism. Both: rate and extent of the dissociation depend, as will be shown here, upon the pH value, temperature, ionic strength as well as ion composition of the medium [1, 10, 11]. All experiments were run under conditions which ensure complete dissociation. In this publication, we will report on the dissociation mechanism, and also on the influence of NaCl concentration on rate, extent, and mechanism of the dissociation.

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Materials and Methods

Partially purified glucose dehydrogenase from *Bacillus megaterium* was kindly supplied by E. Merck, Darmstadt. The enzyme was further purified by stepwise precipitation with ammonium sulfate (J. Hoenes, personal communication).

Enzyme assay

Enzyme activity was determined at 25 °C in a 0.35 M Tris-HCl buffer (pH 8)/3 mM NAD/140 mM glucose, by monitoring the rate of NADH formation at 366 nm.

Ultra-violet absorption

Absorption spectra were measured at 25 °C with a Beckman Acta M VI spectrophotometer in 1-cm pathlength cuvettes. Difference spectra were recorded by means of tandem cuvettes, pathlength 2×0.438 cm. Enzyme concentrations were determined spectrophotometrically with a $A_{1\text{ cm}, 280\text{ nm}}^{1\%}$ value of 9.24 (M_r 120,000).

Circular dichroism

Circular dichroism measurements were performed under temperature control (25 °C) with a Jasco spectropolarimeter J 500 A, equipped with a data processor DP 500 N. At protein concentrations of 0.1–1 mg/ml, the pathlength of the cuvettes was 10–1 cm for measurements in the aromatic region (250 to 350 nm), and 0.1–0.01 cm for the backbone region (185–260 nm). At NaCl concentrations higher than 0.5 M, the circular dichroism backbone spectra could be registered only as far as 195 nm. For the determination of the influence of salts on the protein structure, the spectra were recorded after 30 min incubation in the appropriate buffer solution.

Fluorescence and light scattering

A fluorescence spectrophotometer Perkin-Elmer MPF 44 was employed for the fluorescence and light scattering measurements. Fluorescence emission spectra (excitation at 290 nm) were recorded at 25 °C in the ratio mode, using a 5 nm excitation and 4 nm emission bandwidth, a cuvette pathlength of 1×1 cm, and a protein concentration of 0.1 mg/ml. For light scattering measurements, the emission and excitation monochromators (bandwidth of 5 and 9 nm, respectively) were set to identical wavelengths

(320 or 400 nm). The scattering intensities were measured at a fixed angle of 90°.

In all experiments, the dissociation of the enzyme under "standard conditions" was initiated by adjusting the pH from 6.5 to 9. This pH change was accomplished by diluting a highly concentrated solution of the tetrameric enzyme (10–150 mg/ml in a 67 mM phosphate buffer/0.1–3 M NaCl) into a 50 mM glycine-Tris buffer pH 9. The respective volumes were adjusted so that the final NaCl and KH_2PO_4 concentrations were lower than 10 mM after dilution.

All kinetic measurements were run under these standard conditions at 25 °C. The rate constants were extracted from the first-order plots by way of a graphic approximation procedure [12]. The following equation which is valid in the range of Rayleigh scattering was employed for simulating the light scattering kinetics.

$$I_{90} = H \cdot c \cdot M^2$$

I_{90} denotes the scattered light intensity orthogonally to the direction of irradiation, c the concentration (mol/l) and M the molecular weight of the scattering particles.

H is a function of the index of refraction of the solvent (n_s) and of the refraction index increment at constant potential (dn/dc).

$$H = (12 \pi^2 / \lambda^4 \cdot N_A) \cdot n_s \cdot (dn/dc)^2.$$

In solutions with particles of different molecular weight, as in the case of glucose dehydrogenase dissociation, the total scattered light intensity at the time t is an additive function of contributions from the individual molecular moieties.

$$I_{90}(t) = H_1 \cdot c_1(t) \cdot M_1^2 + H_2 \cdot c_2(t) \cdot M_2^2 + \dots + H_\eta \cdot c_\eta(t) \cdot M_\eta^2.$$

Since solvent conditions do not change appreciably in the course of the dissociation process, a constant value of H for each molecular species (tetramer, dimer and monomer) was assumed in the calculations.

Results

Inactivation

It has been shown previously [1, 10, 11] that by shifting the pH from 6.5 to 9 tetrameric glucose dehydrogenase is dissociated into enzymatically inactive subunits. Complete dissociation and inactivation at pH 9 is assured, however, only at low ionic strengths, *i.e.* in the absence of strong stabilizing

agents such as sodium chloride, phosphate, pyrophosphate or the coenzyme NAD. We have now measured the time dependence of inactivation, at 25 °C, as a function of protein concentration between 0.005 and 1.5 mg/ml under dissociative conditions which meet these requirements (see Methods section) and which also guaranteed a complete reactivation by readjustment of the pH to the original value. The kinetic results show inactivation to be clearly first order with respect to the protein concentration.

The first order plot in Fig. 1A demonstrates that inactivation proceeds by two distinct kinetic phases. The fast phase accounts for about 50% of the activity loss (apparent rate constant $k_1 = 2.56 \times 10^{-2} \text{ s}^{-1}$). For the slower second phase, a value $k_2 = 6.08 \times 10^{-3} \text{ s}^{-1}$ was extracted from the first order plots. Inactivation at 0 °C (Fig. 1B) displayed the same biphasic behaviour, with the rate constants k_1 and k_2 reduced by a factor of 9 and 6.

Light scattering

The time dependence of quaternary structure degradation of glucose dehydrogenase, again under standard dissociative conditions, was studied by light scattering measurements. As Fig. 2A shows, the scattered light intensity decreases rapidly within the first 10 min after initiation of the dissociation. The final value is 28% of the scattered light intensity of the tetrameric enzyme, in good agreement with the 25% value expected for a complete tetramer-monomer conversion. The kinetics again are first order with respect to the enzyme concentration (0.05–1.5 mg/ml). A comparison between the inactivation and the light scattering kinetics demonstrates that degradation of the quaternary structure is markedly retarded relative to enzymic inactivation (Fig. 3). The first order plot (Fig. 2B) for the scattered light intensity kinetics likewise is biphasic. The rate constants for the corresponding two processes ($k_1 = 2.56 \times 10^{-2} \text{ s}^{-1}$, $k_2 = 6.08 \times 10^{-3} \text{ s}^{-1}$) are identical with

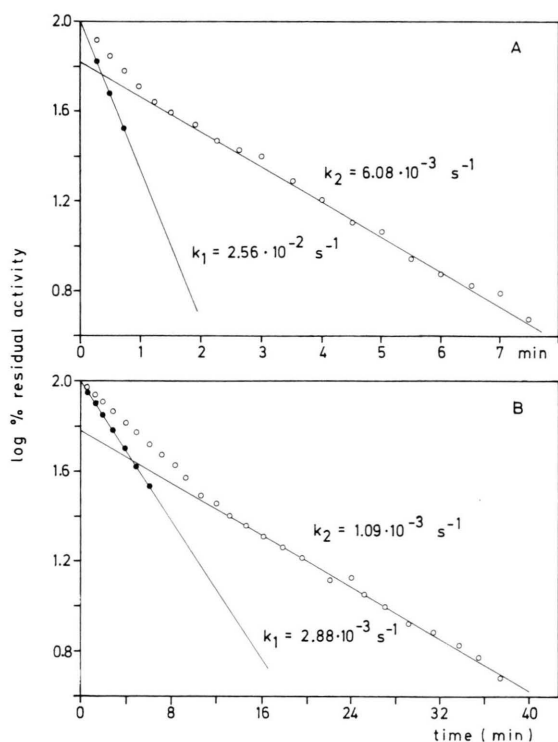


Fig. 1. First-order plots for the rate of inactivation during dissociation in 50 mM glycine-Tris buffer (pH 9), protein concentration 0.1 mg/ml: (A) at 25 °C; (B) at 0 °C.

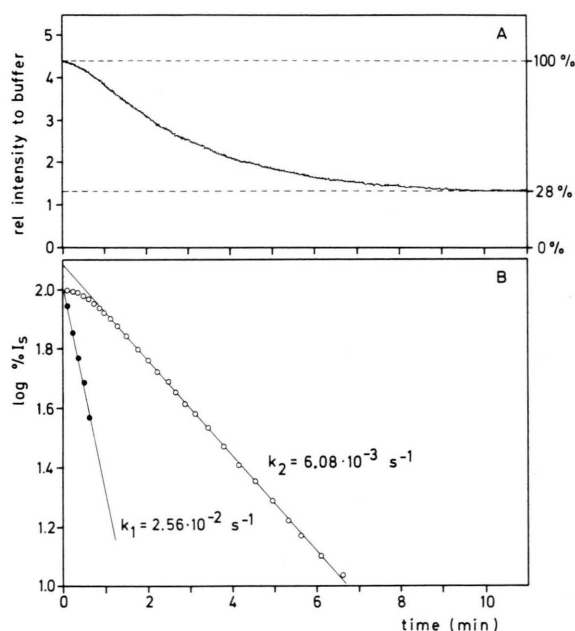


Fig. 2. Time-dependence of light scattering intensity (measured at 320 nm) during dissociation of glucose dehydrogenase at 25 °C and 0.1 mg/ml protein concentration in 50 mM glycine-Tris buffer (pH 9): (A) Zero-order plot; (B) first-order plot.

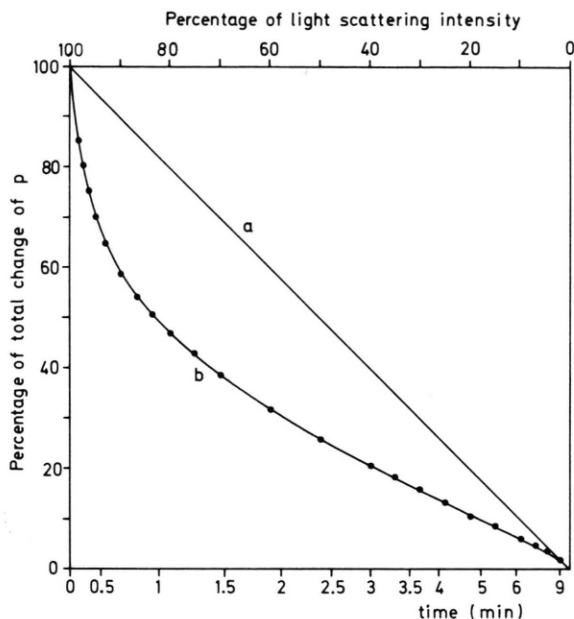


Fig. 3. Correlation of light scattering intensity (in percent of total change during dissociation) versus percent total change of enzyme activity. Dissociation was performed at 25 °C and 0.1 mg/ml protein concentration in 50 mM glycine-Tris buffer (pH 9): (a) Light scattering intensity; (b) enzyme activity.

those determined for the inactivation kinetics. The faster of the two processes which accounts for about 50% of the inactivation is responsible for the lag phase in the light scattering kinetics. Consequently, this process must involve an internal rearrangement of the protein structure, prior to the actual degradation of the quaternary structure which is the slower of the two processes ($k_2 = 6.08 \times 10^{-3} \text{ s}^{-1}$).

Conformational changes

Extensive spectroscopic investigations [1] have demonstrated beyond any doubt that the dissociation process "releases" tryptophan moieties from either the protein core or the subunit contact region, thus exposing these chromophores to direct contact with the solvent medium. By following the time dependence of the ultra-violet absorption at 232, 284 and 292 nm, of the circular dichroism signals at 232 and 300 nm, and of the fluorescence intensity at 333 nm (for the actual spectra see ref. [1]), we tried to get some insight into the structural changes in the micro-environment of the respective tryptophan moieties.

We also hoped to show by these measurements to which extent these structural changes are connected with the degradation of the quaternary structure or with a structural rearrangement of glucose dehydrogenase itself.

All kinetic measurements were run under the standard dissociation conditions and displayed not the slightest dependence on enzyme concentration in the range 0.005–1.5 mg/ml (see Fig. 4). Kinetic analysis of the corresponding first order plots two of which are shown in Fig. 5, established the presence of three distinct mechanistic phases (Table I): the two slower processes which were already known from the light scattering and inactivation kinetics, are preceded by an additional jump in the intensity of the tryptophan signals immediately after initiation of the dissociation. Since the dissociation is actually triggered by a rapid change in pH, this fast process could represent a charge perturbation of tryptophan moieties on the protein surface (ionization of neighbouring groups; as a matter of fact, one of the four

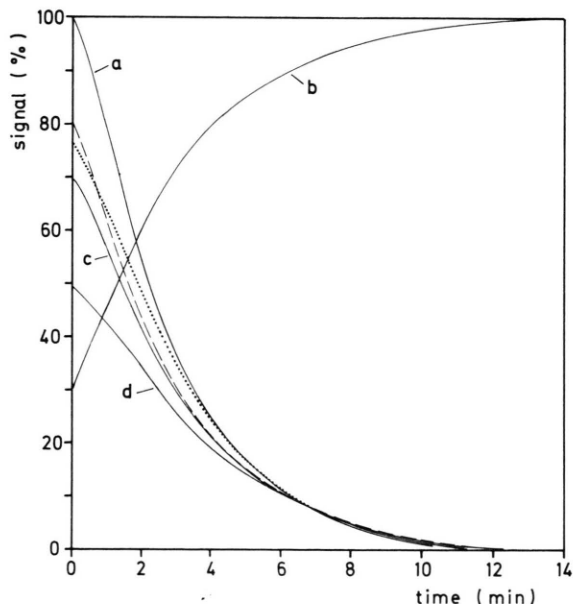
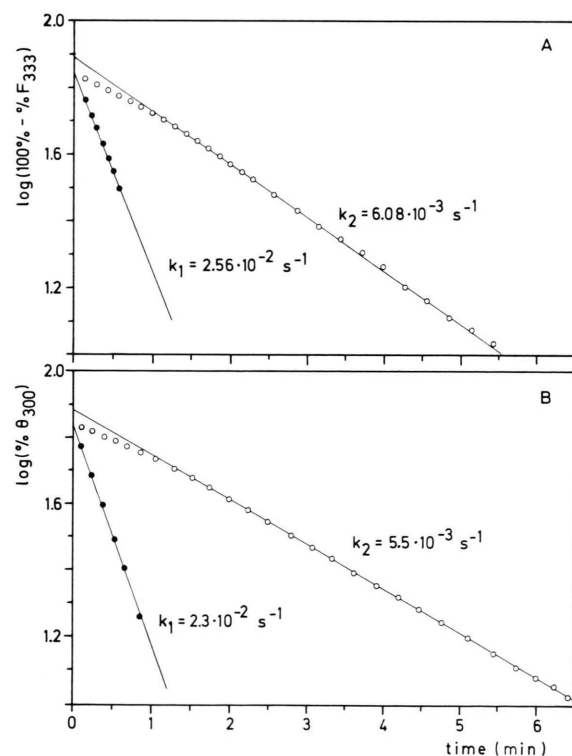


Fig. 4. Time-dependence of various spectroscopic responses from the intrinsic tryptophan residues of glucose dehydrogenase during dissociation (at 25 °C and 0.1 mg/ml protein concentration in 50 mM glycine-Tris buffer (pH 9)): (a) Ultra-violet absorption at 232 nm; (b) fluorescence intensity at 333 nm; (c) circular dichroism at 300 nm; (d) circular dichroism at 232 nm; (---) ultra-violet absorption at 294 nm; (····) ultra-violet absorption at 284 nm.

Table I. Kinetic parameters for the dissociation of glucose dehydrogenase, obtained by various spectroscopic methods. Dissociation was performed at 25 °C and 0.1 mg/ml protein concentration in 50 mM glycine-Tris buffer (pH 9). The rate constants for the individual processes, as well as the extent of the individual decays, were derived from first-order plots and/or by mathematical simulation.

Kinetics	1. Process		2. Process		3. Process	
	Rate constant k_0	Extent [%]	Rate constant k_1 $\times 10^{-3} \text{ s}^{-1}$	Extent [%]	Rate constant k_2 $\times 10^{-3} \text{ s}^{-1}$	Extent [%]
Enzyme activity	—	—	2.56	50	6.08	50
Light scattering 320 nm	—	—	2.56	0	6.08	100
Fluorescence intensity 333 nm	92.4 s^{-1*}	30	2.56	10	6.08	60
Circular dichroism 300 nm	fast	30	2.30	7	5.50	63
Circular dichroism 232 nm	fast	50	1.92	4	5.25	46
Ultra-violet absorption 232 nm	—	—	2.3	20	6.08	80
Ultra-violet absorption 284 nm	fast	23	1.95	8	6.24	69
Ultra-violet absorption 292 nm	fast	19	2.88	12	6.08	69

* Stopped-flow measurement.



tryptophan residues per subunit is accessible on the surface of the tetramer (K.-D. Jany, W. List, personal communication). Time-resolution of this jump phase by stopped-flow fluorescence measurements (Fig. 6 A, B), however, established first order kinetics for this process ($t_{1/2} = 7.5 \text{ ms}$, corresponding to a rate constant $k_0 = 92.4 \text{ sec}^{-1}$). This rate is by far too low for a deprotonation reaction, *i.e.* a pH effect [13].

We rather surmise that this process represents a rapid structural rearrangement in the close vicinity of the tryptophan chromophores, induced by ionization of the protein surface. It remains open whether additional fast processes, such as the charge perturbation described above, contribute to the jump phase; likewise unexplained is the missing jump phase in the ultra-violet kinetics (232 nm, see ref. [1]).

Fig. 5. First-order plots for the rates of change of spectroscopic signals from the intrinsic tryptophan residues of glucose dehydrogenase during dissociation (conditions as given for Fig. 4): (A) Fluorescence intensity at 333 nm (excitation at 290 nm); (B) circular dichroism at 300 nm.

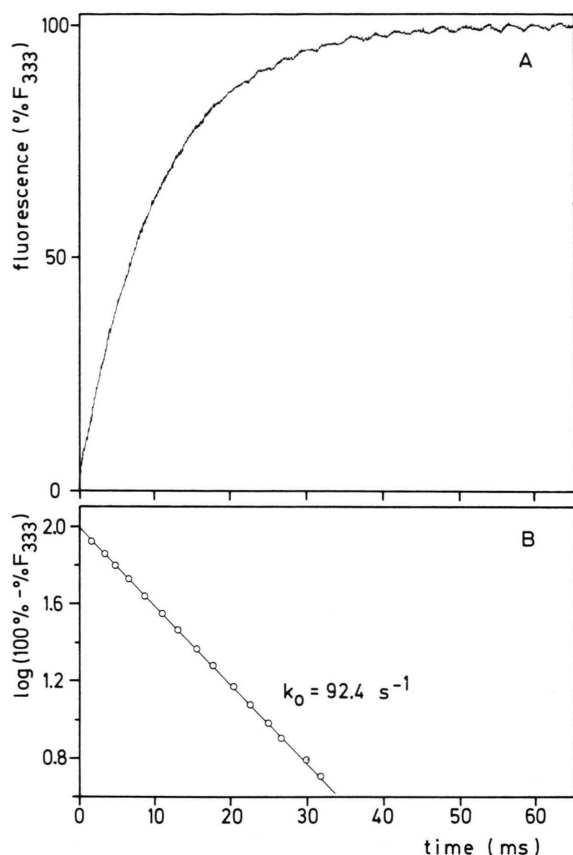


Fig. 6. Stopped-flow fluorescence kinetics at 333 nm (excitation at 290 nm). Dissociation was performed at 25 °C and 0.5 mg/ml protein concentration in 50 mM glycine-Tris buffer (pH 9): (A) Zero-order plot; (B) first-order plot.

Influence of NaCl on kinetics and mechanism of the dissociation

As reported [2], structure and stability of glucose dehydrogenase, and hence the dissociation and re-association processes, are influenced by both: the ionic strength and ion composition of the buffer. Increasing NaCl concentration lowers rate as well as extent of the dissociation as the fluorescence kinetics demonstrate (see Fig. 7). The first order plot in Fig. 8A shows, however, no fundamental differences between dissociation in the presence of 100 mM NaCl and under standard conditions, *e.g.* without any NaCl (*cf.* Fig. 5A). Apart from a jump phase which accounts for about 30% of the total change in intensity, two processes become once more apparent; their

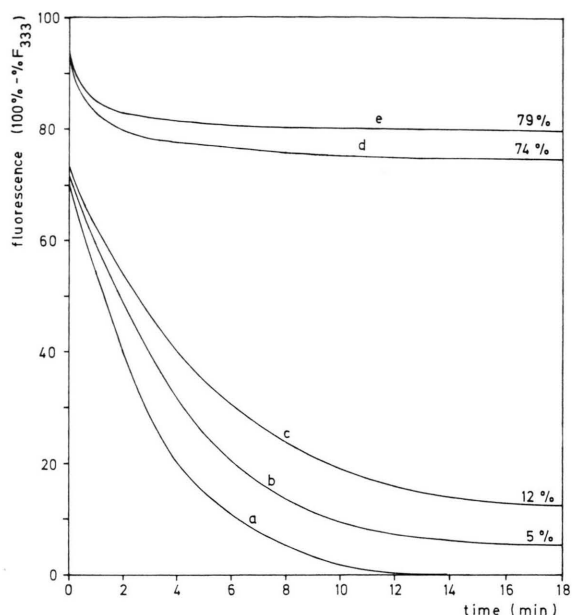


Fig. 7. Influence of NaCl on rate and extent of dissociation at 25 °C. Decay of fluorescence intensity at 333 nm (excitation at 290 nm) during dissociation of 0.1 mg/ml glucose dehydrogenase in 50 mM glycine-Tris buffer (pH 9): (a) 0 and 10 mM NaCl; (b) 50 mM NaCl; (c) 100 mM NaCl; (d) 500 mM NaCl; (e) 500 mM NaCl, protein concentration raised to 0.6 mg/ml. The intensity change for complete dissociation in the absence of NaCl (curve a) was set as 100%.

rate, though, is slightly lowered due to the influence of the NaCl ($k_1 = 9.6 \times 10^{-3} \text{ s}^{-1}$, $k_2 = 3.6 \times 10^{-3} \text{ s}^{-1}$). The fluorescence kinetics remain first order with respect to enzyme concentration; the total change in intensity, while still independent of enzyme concentration, appears reduced by about 10% as compared to complete dissociation (see Fig. 7). For a dissociation in the presence of 500 mM NaCl, on the other hand, the kinetics definitely obey a higher order; rate (Fig. 8B) as well as extent of the dissociation (Fig. 7) now strongly depend on the enzyme concentration. These results definitely indicate the establishment of a dissociation/association equilibrium. By sedimentation equilibrium studies in the analytical ultracentrifuge, we could show [14] that glucose dehydrogenase dimers, besides monomers and tetramers, participate in aggregation equilibria which in this manner depend on enzyme and NaCl concentration, and on the pH value.

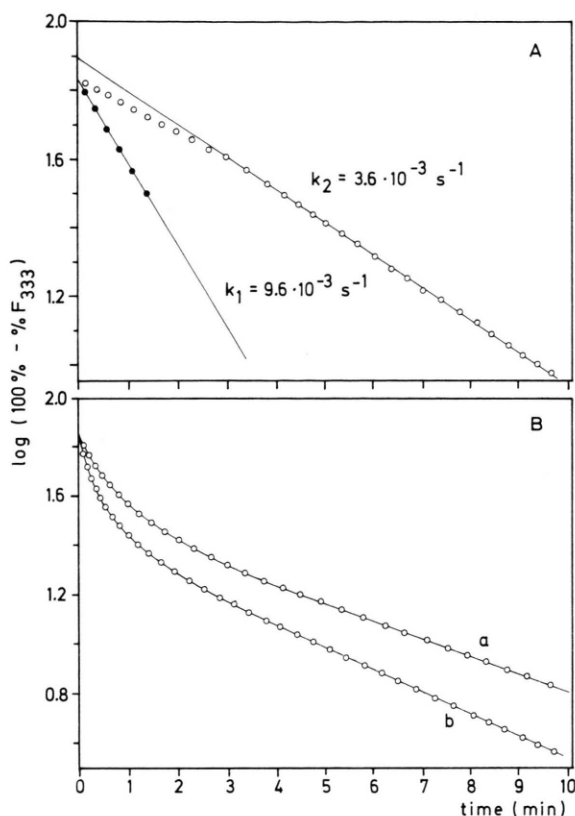
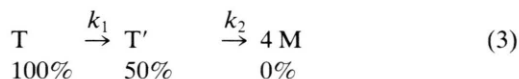
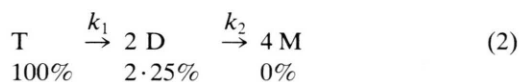
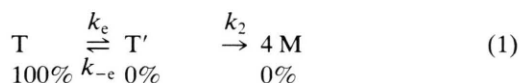


Fig. 8. Influence of NaCl on the dissociation kinetics of glucose dehydrogenase at 25 °C in 50 mM glycine-Tris buffer (pH 9), monitored by the change in fluorescence intensity at 333 nm (excitation at 290 nm): (A) Dissociation in the presence of 100 mM NaCl, with the protein concentration varying from 0.1 to 0.6 mg/ml; (B) dissociation in the presence of 500 mM NaCl at a protein concentration of (a) 0.1 mg/ml and (b) 0.6 mg/ml.

Discussion

The kinetic results summarized in Table I show the overall dissociation process to be composed of at least three distinct, rapid first order steps. The first, extremely fast reaction $k_0 = 92.4 \text{ s}^{-1}$ can be observed only in the spectroscopic kinetics of the tryptophan signals. This process which is not connected with a change in either molecular size or enzymic activity must therefore represent a rapid structural rearrangement within the active tetramer. The two subsequent, slower processes are observed in the time-dependence of all investigated variables, and are characterized by average first order rate constants $k_1 = 2.56 \times 10^{-2} \text{ s}^{-1}$ and $k_2 = 6.08 \times 10^{-3} \text{ s}^{-1}$, respec-

tively. The stepwise reduction of the enzymic activity by these two processes (50% each) indicates that there is a further dissociation intermediate present. This intermediate could be either a tetramer with 50% specific activity, or the glucose dehydrogenase dimer (*i.e.* two moieties with 25% activity each), or a completely inactive product which exists in equilibrium with fully active tetramer. Since all the individual processes are concentration-independent, the following mechanistic alternatives can be envisaged:



T/D/M/T' denote native tetramer/dimer/monomer/rearrangement tetramer, respectively; $k_1 = 2.56 \times 10^{-2} \text{ s}^{-1}$, $k_2 = 6.08 \times 10^{-3} \text{ s}^{-1}$, $k_e = k_{-e} = \frac{1}{2}(k_1)$; the percentage values give the relative enzymic activities.

A computer simulation of the activity change, expected if mechanism (1) held, gave no agreement at all with the experimentally determined inactivation kinetics (see Fig. 9A). The experimentally determined value for k_1 was employed for the resulting rate constant of equilibrium establishment (which is composed, additively, from the rate constants of the for- and backward reaction, k_e/k_{-e}). Non-fit could be obtained even if various equilibrium positions (*i.e.* k_e/k_{-e} quotients) were employed.

On the basis of the mechanism (2), an activity time-dependence is calculated which is in perfect agreement with the experimental inactivation kinetics (see Fig. 9A); the corresponding simulation of the light scattering kinetics (Fig. 9B), on the other hand, exhibits drastic discrepancies from the experimental curve. A dimer with 50% specific activity can thus be ruled out as intermediate for the glucose dehydrogenase dissociation.

With the experimentally determined values for the rate constants k_1 and k_2 , and by assuming a tetramer with reduced specific activity ($50 \pm 5\%$) as intermediate (mechanism (3)), a perfect match is obtained between calculated and experimental inactivation and light scattering intensity (Fig. 9A,B).

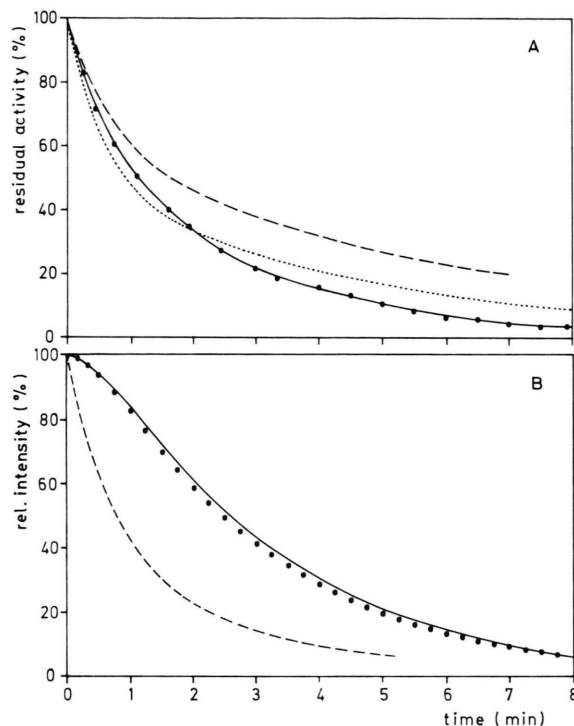


Fig. 9. A: Simulation of the inactivation kinetics in the course of glucose dehydrogenase dissociation at 25 °C in 50 mM glycine-Tris buffer (pH 9): (····) Experimentally determined time dependence of activity; (----) time dependence, calculated on the basis of mechanism (1) with $k_e = k_{-e} = k_{1/2} = 1.28 \times 10^{-2} \text{ s}^{-1}$, $k_2 = 6.08 \times 10^{-3} \text{ s}^{-1}$, relative enzyme activities T, T' and M taken as 100, 0 and 0%, respectively; (····) calculated on the basis of mechanism (1) with $k_e/k_{-e} = 1.75$, $k_e = 1.63 \times 10^{-2} \text{ s}^{-1}$, $k_{-e} = 9.3 \times 10^{-3} \text{ s}^{-1}$, $k_2 = 6.08 \times 10^{-3} \text{ s}^{-1}$, relative enzyme activities T, T' and M taken as 100, 0 and 0%, respectively; (—) calculated on the basis of mechanism (2) and (3) both being operative, with $k_1 = 2.56 \times 10^{-2} \text{ s}^{-1}$, $k_2 = 6.08 \times 10^{-3} \text{ s}^{-1}$, relative enzyme activities T, T', D and M taken as 100, 50, 25 and 0%, respectively.

B: Simulation of the light scattering kinetics (320 nm) in the course of glucose dehydrogenase dissociation at 25 °C in 50 mM glycine-Tris buffer (pH 9): (····) Experimentally determined time dependence of light scattering intensity; (----) time dependence, calculated on the basis of mechanism (2) with $k_1 = 2.56 \times 10^{-2} \text{ s}^{-1}$, $k_2 = 6.08 \times 10^{-3} \text{ s}^{-1}$; (—) calculated on the basis of mechanism (3) with $k_1 = 2.56 \times 10^{-2} \text{ s}^{-1}$, $k_2 = 6.08 \times 10^{-3} \text{ s}^{-1}$.

Summarizing, the following three-step mechanistic sequence can be established for the pH-induced dissociation of glucose dehydrogenase (Fig. 10): the pH

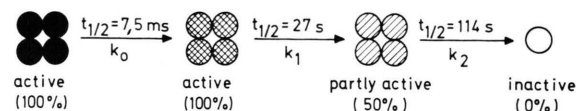


Fig. 10. Mechanism of glucose dehydrogenase dissociation at 25 °C and pH 9 with $k_0 = 92.4 \text{ s}^{-1}$, $k_1 = 2.56 \times 10^{-2} \text{ s}^{-1}$, $k_2 = 6.08 \times 10^{-3} \text{ s}^{-1}$.

change induces charge perturbations on the protein surface which in turn trigger a rapid structural rearrangement of the tetramer which does not influence its enzymic activity. This "induction phase" is followed by a second, much slower rearrangement which is responsible for a significant reduction of specific activity. The actual dissociation, *i.e.* the dissolution into inactive monomers, follows only as the third step. On the basis of kinetic simulations, a dimeric intermediate can be excluded for this dissociation sequence.

Monomer and tetramer of glucose dehydrogenase differ practically only in their respective tertiary structures. Table I shows which changes in tertiary structure correspond to the individual mechanistic steps of the dissociation. The gravest structural changes in the micro-environment of the tryptophan chromophores occur during the actual dissolution step in which groups participating in subunit contact in the native enzyme are exposed to the solvent medium. Modification experiments [15] support the mechanism rationale outlined here.

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