for ODH, as shown in kinetic studies (Doublet et al., in preparation), some of the studied complexes are involved in the regulation mechanism. Such heterogeneity might also be present in the evolutive complexes and play a direct role in the catalytic process.

References

Baici, A., Luisi, P.-L., Olomucki, A., Doublet, M.-O., and Klincak, J. (1974), *Eur. J. Biochem. 46,* 59.

Biellmann, J.-F., Branlant, G., and Wallen, L. (1977), *Bioorg. Chem. 6* (in press).

Brochon, J.-C., Wahl, Ph., Jallon, J. M., and Iwatsubo, M. (1976), *Biochemistry 15,* 3259.

Donzel, B., Gauduchon, P., and Wahl, Ph. (1974), *J. Am. Chem. Soc. 94,* 801–808.

Doublet, M.-O., and Olomucki, A. (1975), *Eur. J. Biochem. 59,* 175.

Gafni, A., and Brand, L. (1976), *Biochemistry 15,* 3165.

Huc, C., Olomucki, A., Lan, L. T., Pho, D. B., and Thoai, N. v. (1971), *Eur. J. Biochem. 21,* 161.

Jablonski, A. (1961), *Z. Naturforsch., B 16,* 1.

Luisi, P.-L., Baici, A., Olomucki, A., and Doublet, M.-O. (1975), *Eur. J. Biochem. 50,* 511.

Luisi, P.-L., Olomucki, A., Baici, A., and Karlovic, D. (1973), *Biochemistry 12,* 4100.

Ohlsson, J., Nordström, B., and Bränden, C.-J. (1974), *J. Mol. Biol. 89,* 339.

Olomucki, A., Huc, C., Lefébure, F., and Thoai, N. v. (1972), *Eur. J. Biochem. 28,* 261.

Olomucki, A., Thomé-Beau, F., Biellmann, J. F., and Branlant, G. (1975), *Eur. J. Biochem. 56,* 109.

Oriol, C., and Olomucki, A. (1972), *Eur. J. Biochem. 29,* 288.

Pho, D. B., Olomucki, A., Huc, C., and Thoai, N. v. (1970), *Biochim. Biophys. Acta 206,* 46.

Schutte, E. (1943), *Hoppe-Seyler's Z. Physiol. Chem. 279,* 52.

Thoai, N. v., Huc, C., Pho, D. B., and Olomucki, A. (1969), *Biochim. Biophys. Acta 191,* 46.

Thoai, N. v., and Robin, Y. (1961), *Biochim. Biophys. Acta 52,* 221.

Thomé-Beau, F., and Olomucki, A. (1973), *Eur. J. Biochem. 39,* 557.

Wahl, Ph. (1969), *Biochim. Biophys. Acta 175,* 55.

Wahl, Ph. (1975), *New Tech. Biophys. Cell Biol. 2,* 233.

Wahl, Ph., and Auchet, J. C. (1972), *Biochim. Biophys. Acta 285,* 99.

Wahl, Ph., Auchet, J. C., and Donzel, B. (1974), *Rev. Sci. Instrum. 45,* 28.

Yguerabide, J., Epstein, H. F., and Stryer, L. (1970), *J. Mol. Biol. 51,* 573.

Conformational and Functional Aspects of the Reversible Dissociation and Denaturation of Glucose Dehydrogenase†

Hans E. Pauly and Gerhard Pfleiderer*

ABSTRACT: Glucose dehydrogenase from *Bacillus megaterium* represents a stable tetramer at pH 6.5 but was readily dissociated into four inactive protomers by shifting the pH to 8.5. Complete and rapid reassociation and reactivation could be achieved by readjustment to the original pH value. High concentrations of NaCl and the presence of the coenzyme NAD stabilize the quaternary structure. Unfolding of the enzyme in 8 M urea is also strongly inhibited by high concentrations of NaCl, probably due to a conformational transition induced by the salt. Complete reactivation of unfolded GlucDH can be achieved via a monomeric intermediate obviously identical with that state obtained by dissociation of the enzyme at pH 9.0. Optical rotatory dispersion spectra and the immunological reactivity of the tetrameric and dissociated enzyme revealed no differences. Dissociation promotes a blue shift of the ultraviolet absorption, probably due to the exposure of one tryptophyl residue to the solvent, a decrease of the polarization of intrinsic fluorescence and the exposure of hydrophobic areas of the enzyme surface which may be concluded from the increase of the number of binding sites for 8-anilino-1-naphthalenesulfonate. Inactivity of the subunit is due to the loss of the coenzyme binding capacity. The correlation between functional and conformational changes during dissociation emphasizes the close connection between changes at the active site and the intersubunit binding domains of the enzyme.

GlucDH catalyses the oxidation of β-D-glucose to D-glucono-1,5-lactone using NAD or NADP as coenzyme. The purification of this enzyme from *Bacillus megaterium* M 1286, its application for the quantitative determination of glucose in biological substances, and some of its properties have recently been reported (Banauch et al., 1975; Pauly & Pfleiderer, 1975). This dehydrogenase represents a tetramer (molecular weight 118 000) consisting of polypeptide chains identical in size and charge and shows the unusual ability of a completely reversible dissociation to the protomers under very mild conditions.

There exists no general answer to the question whether catalytic activity of multisubunit enzymes is restricted to the oligomeric form. To our knowledge, however, in no case active monomers could be confirmed for dehydrogenases (Chan &

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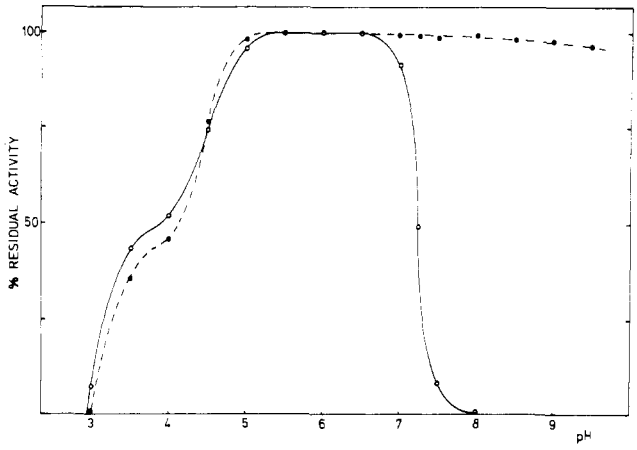

FIGURE 1: pH-induced inactivation and reactivation of GlucDH. (○) Residual activity of 5.2×10^{-7} M GlucDH after inactivation at various pH values for 30 min. Buffers, 50 mM were used: formate, acetate, imidazole, Tris, glycine. (●) Activity recovery of GlucDH dissociated as described in ○ measured 45 min after the beginning of the routine reassociation procedure.

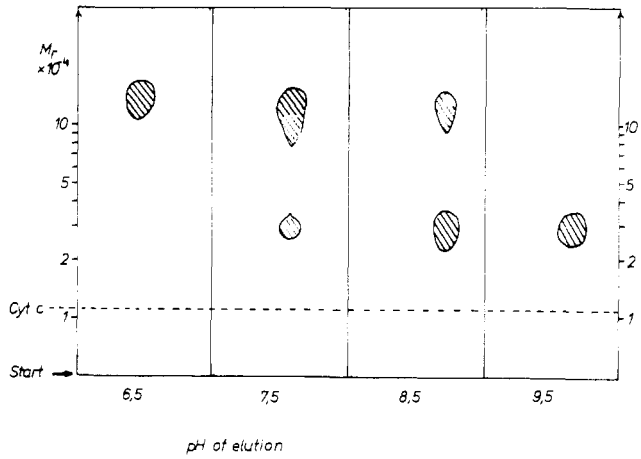

FIGURE 2: Thin-layer gel chromatography of samples of native GlucDH (2.5×10^{-5} M) using elution buffers of different pH and 50 mM NaCl–50 mM imidazole-HCl buffer, pH 6.5, 50 mM Tris-HCl buffer, pH 7.5 and 8.5, 50 mM glycine–Tris buffer, pH 9.5. Detection of monomeric and tetrameric GlucDH by specific staining (Pauly & Pfleiderer, 1975). Migration was determined in relation to that of cytochrome *c* using ovalbumin, serum albumin, and lactate dehydrogenase as reference proteins of known molecular weight.

Mosbach, 1976; Rudolph & Jaenicke, 1976). Because of the easiness and complete reversibility of the dissociation of GlucDH, the conformational changes leading to the loss of quaternary structure and inactivation should be small and reflect the close dependence of subunit interactions and enzyme activity. Therefore the effect of the reversible dissociation of tetrameric GlucDH to the protomers on the conformational and functional properties has been studied.

Rate and extent of dissociation depend on pH, temperature, ionic strength, ionic composition of the medium, and on protein concentration. Details will be described in a following paper. Experiments reported here were performed under conditions which guaranteed that the enzyme existed either completely in its tetrameric or its monomeric form.

Materials and Methods

GlucDH[1] was purified from *Bacillus megaterium* M 1286 as previously described (Pauly & Pfleiderer, 1975) using a partially purified commercial preparation from E. Merck, Darmstadt. The enzyme was routinely stored in 67 mM phosphate buffer, pH 6.5, containing 3 M NaCl and dialyzed prior to use against 33 mM phosphate buffer, pH 6.5, containing 0.1 M NaCl.

The performance of the standard enzyme assay used in all experiments, disc-gel electrophoresis, isoelectric focusing, and density gradient centrifugation has also been described (Pauly & Pfleiderer, 1975). Complete dissociation of GlucDH was routinely achieved by incubation of the enzyme (0.01–0.2 mg/mL) for 30 min at 25 °C with 50 mM glycine–Tris buffer, pH 9.0, containing 50 mM NaCl. Reactivation was started by the addition of equal volumes of reassociation buffer leading to a final concentration of 0.5 M phosphate buffer, pH 6.5, 10 mM EDTA, 1 M NaCl, and 0.5 mg/mL bovine serum albumin. When serum albumin was omitted, reactivation was performed in very dilute solutions of enzyme (<0.01 mg/mL) which were concentrated prior to spectroscopic and electrophoretical investigations.

Renaturation of GlucDH was achieved as described by Deal (1969) by dialysis or dilution of samples incubated 1 h at 0 °C in 33 mM phosphate buffer, pH 7.2, containing 8 M urea and 50 mM EDTA.

A Cary 60 polarimeter was used for measurements of the optical rotatory dispersion in 1-cm pathlength cuvettes. Protein concentration ranged from 0.15 to 0.35 mg/mL.

Absorption difference spectra were measured at 25 °C in a Beckman 25 recording spectrophotometer. Fluorescence measurements were performed at 25 °C with a Perkin-Elmer fluorescence spectrophotometer MPF-44 using polarization accessory 018-0054. The spectrofluorimeter was operated in the ratio-recording option using an excitation bandwidth of 1 nm and an emission bandwidth of 20 nm.

Results

Correlation of Enzymatic Activity and Dissociation. At pH 6.5 in the presence of high concentrations of NaCl, GlucDH shows optimal stability against thermal inactivation, and solutions of the enzyme may be stored for months at room temperature without loss of activity. In the absence of NaCl moderately changing the hydrogen ion concentration above or below the point of neutrality results in a fast decrease of activity (Figure 1). Reversal of the pH to original value leads to a complete restoration of enzymatic activity only in the case of the alkali-inactivated enzyme, whereas precipitation and irreversible inactivation are observed for GlucDH incubated below pH 5.

Inactivation of GlucDH in weak alkaline solutions coincides with the dissociation of the native tetrameric enzyme to subunits, their molecular weight corresponding to that of the single polypeptide chains. Decreasing the hydrogen ion concentration of the separation medium increasing amounts of monomeric GlucDH may be observed either in thin-layer gel chromatography (Figure 2), disc gel electrophoresis (Pauly & Pfleiderer, 1975), or in density gradient centrifugation according to Martin & Ames (1961). The sedimentation coefficient $s_{20,w}$ of the monomer was calculated to be 3 S, that of the tetramer 6, 5 S using lactate dehydrogenase and human serum albumin, labeled with 2,4-dinitrobenzene, as reference proteins.

Distinct bands of monomeric and tetrameric enzyme could be observed in these experiments. It must be assumed that

[1] Abbreviations used are: GlucDH, glucose dehydrogenase β-D-glucose:NAD(P)$^+$ 1-oxidoreductase (EC 1.1.1.47); EDTA, ethylenediaminetetraacetic acid; Tris, tris(hydroxymethyl)aminomethane; UV, ultraviolet.

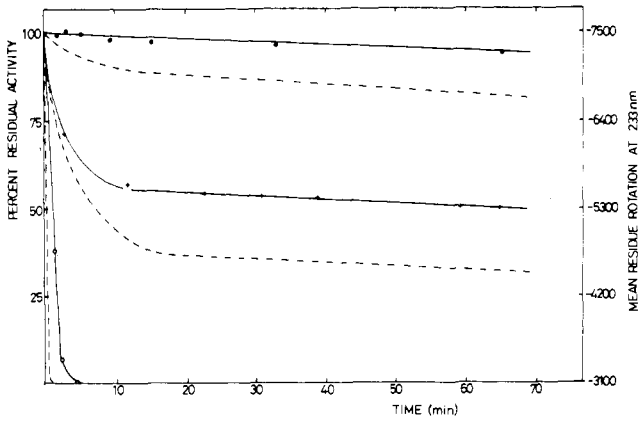

FIGURE 3: Inactivation and optical rotation of 5.2×10^{-7} M GlucDH in 8 M urea. Enzymatic activity (—); optical rotation at 233 nm (- - -). (○) No addition; (+) addition of 1 M NaCl to the incubation mixture; (●) incubation of GlucDH in a solution of 1 M NaCl for 30 min prior to the exposure to 8 M urea containing 1 M NaCl.

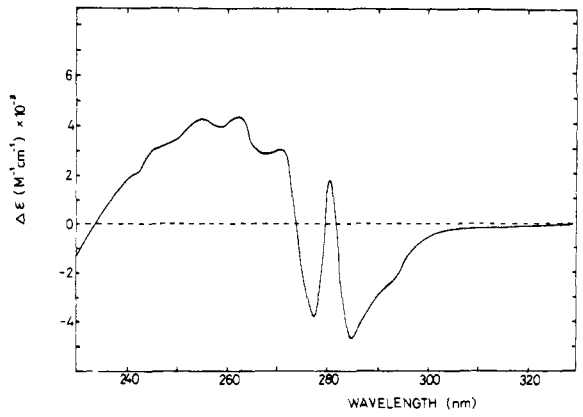

FIGURE 4: Absorption difference spectrum of monomeric (100 mM glycine–Tris buffer, pH 9.0, and 50 mM NaCl) minus tetrameric GlucDH (8 mM phosphate buffer, pH 6.5, and 50 mM NaCl); protein concentration 0.3 mg/mL.

under these conditions, e.g., high protein concentration and ionic composition of the separating medium, in spite of alkaline media, the time required to attain the equilibrium of dissociation is large compared with the duration of the experiments.

Reversible Denaturation of GlucDH. Unfolding of the enzyme in 8 M urea in the absence or in the presence of low concentrations of NaCl is completed in less than 2 min as monitored by the change in optical rotation at 233 nm. Addition of 1 M NaCl prior to the exposure of the enzyme to urea strongly inhibits the unfolding by urea as shown in Figure 3. About 90% of the optical activity of native GlucDH at 233 nm is left 30 min after the addition of urea and the half-time of inactivation increases by a factor of about 500. Omitting the preincubation period with 1 M NaCl results in a fast initial decrease of activity until the inactivation rate approaches the value of the pretreated enzyme emphasizing the time dependence of the stabilizing action of NaCl. The substrates to which is often attributed a stabilizing effect against urea denaturation as in the case of glucose dehydrogenase from *Bacillus cereus* (Sadoff et al., 1965) fail in preventing or slowing down inactivation.

Renaturation of unfolded GlucDH restored more than 90% of the original specific activity 60 min after dilution and after complete dialysis, respectively.

The optimum of temperature for refolding was in the range of 25 °C. The initial rate of reactivation at higher temperatures exceeded that observed at 25 °C, but the extent of final reactivation was greatly reduced. A similar observation has been reported for aldolase resulting from the appearance of a heat labile monomeric intermediate during the refolding of the enzyme (Vimard et al., 1975).

No difference in rate and extent of reactivation could be detected by using direct dialysis against reassociation buffer or alternatively a two-step procedure, consisting of dialysis against 50 mM glycine–Tris buffer, pH 9.0, and consecutive dialysis against reassociation buffer. The product of the first step is inactive but exhibits an optical rotation at 233 nm identical with that of the native enzyme. The behavior of this refolded GlucDH in disc-gel electrophoresis, isoelectric focusing, and in thin-layer gel chromatography, however, is identical with that of monomeric GlucDH, which obviously represents an intermediate in the complete reactivation of unfolded GlucDH.

Conformational Characteristics of Tetrameric and Monomeric Glucose Dehydrogenase. Optical rotatory dispersion curves in the range of 220–320 nm were recorded for the native, the dissociated, and the reassociated enzyme. The curves were superposable, all displaying the characteristic negative Cotton effect with a minimum trough between 232 and 233 nm and mean residue rotations at these wavelengths of −7500. These experiments reveal that no dramatic changes accompany the dissociation of glucose dehydrogenase leading to the inactive protomers. These results could be confirmed by the investigation of the immunological reactivity of the native, monomeric, and the reconstituted enzyme. Immunological identity was proved by double immunodiffusion according to Ouchterlony (1948) and the radial immunodiffusion technique of Mancini et al. (1965) using an anti-tetramer directed rabbit antiserum. As this antiserum failed to induce reactivation of monomeric GlucDH the antigenic determinants remain unchanged in all forms of the enzyme.

Indirect evidence for the disruption of the quaternary structure comes from the UV-difference spectrum of the dissociated versus the tetrameric enzyme. The spectrum shown in Figure 4 reflects that alterations of the microenvironment of aromatic residues take place during dissociation. A blue shift is observed in the region from 285 to 300 nm arising from a changed absorption of tryptophyl residues. The positive region of the spectrum below 285 nm shows minor contributions of tyrosyl and phenylalanyl residues besides a positive contribution of tryptophan which has to be expected in this region (Yanari and Bovey, 1960). The usefulness of tryptophyl residues as reporter groups for conformational changes in GlucDH is also evidenced by the intrinsic fluorescence polarization spectra shown in Figure 5. Conditions which stabilize the tetrameric structure, e.g., high concentrations of NaCl at pH 6.5, result in a large polarization of fluorescence which may be due to a tightening of the enzyme structure by charge–charge interactions. This rigidity of the macromolecule may also be responsible for the stability of GlucDH against heat and urea inactivation under these conditions (Sadoff et al., 1965).

Low concentrations of NaCl at pH 6.5 and the rise of pH in the presence of 1 M NaCl, respectively, lead to a marked decrease of polarization without influencing enzymatic activity and the quaternary structure. Dissociation to the subunits at pH 8.5 results in a further decrease of polarization and a shift of the characteristic peak at 290 nm to 287 nm reflecting the enhanced flexibility of the protomers and the solvent perturbation of tryptophan in the monomeric state.

Concomitant to the decrease of fluorescence polarization

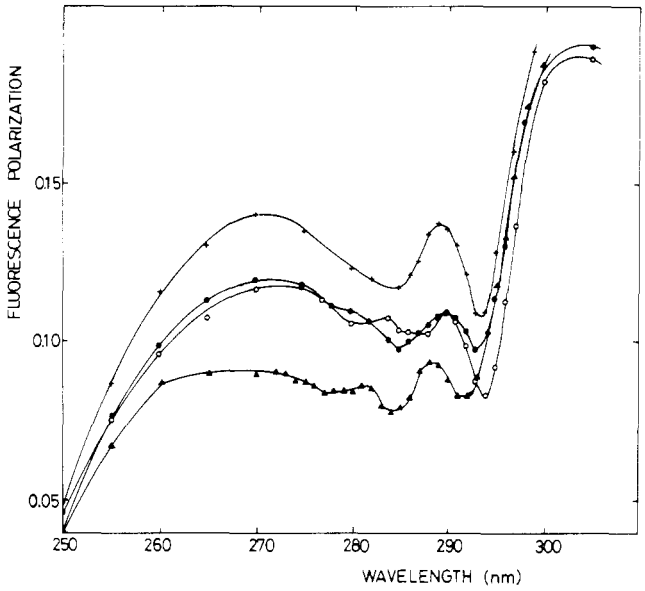

FIGURE 5: Fluorescence polarization spectra of 5.2×10^{-7} M GlucDH at 25 °C; emission at 330 nm. With 33 mM phosphate buffer, pH 6.5, and 1 M NaCl (+), or with 50 mM NaCl (○); 50 mM glycine-Tris buffer, pH 9.0, and 1 M NaCl (●); or with 50 mM NaCl (▲). The polarization of fluorescence was measured 30 min after the dilution of the enzyme stock solution with the buffers indicated above.

TABLE I: Fluorescence Titrations with 8-Anilino-1-naphthalenesulfonic Acid.[a]

Conditions corresponding to Figure 5	No. of binding sites per subunit	Dissociation constant (M)	Rel fluorescence intensity	Enzymatic act. (%)
a	1.2	1.2×10^{-5}	0.22	100
b	2.1	7.4×10^{-6}	0.41	100
c	2.0	1.8×10^{-5}	0.45	95
d	3.2	2.1×10^{-5}	1.73	3

[a] Increasing amounts of the dye were added to a solution of 1.4×10^{-6} M GlucDH. Fluorescence intensity was recorded (excitation 390 nm; emission 470 nm). Computation was performed with a Wang 2200/2002 computer using an interative curve fitting procedure according to Engel (1974).

there is an increase of the number of binding sites for 8-anilino-1-naphthalenesulfonate. The results of the fluorescent titration of GlucDH with 8-anilino-1-naphthalenesulfonate are presented in Table I, using conditions identical with those described in Figure 5. The number of binding sites and the binding constants were computed using a procedure for an iterative curve fitting to a theoretical binding hyperbola described by Engel (1974). The inducement of a less rigid structure increased the number of binding sites by one. Another additional site was created during the dissociation of the enzyme to the protomers. The presence of the dye did not influence the equilibrium of dissociation and no competition between the fluorescent reagent and the coenzyme NAD for one of the binding sites could be observed.

The relative intensity of fluorescence which represents a measure for the hydrophobicity of the binding site doubles during the conformational transition of the tetramer but is increased fourfold during dissociation. This marked enhancement of fluorescence emphasizes the hydrophobic character of the intersubunit binding domains of the enzyme.

Functional Differences between Tetrameric and Monomeric Glucose Dehydrogenase. As described in Figure 1, dissociation leads to a complete but reversible inactivation of GlucDH. This loss of catalytic function is due to the loss of the coenzyme binding capacity. The number of the coenzyme binding sites of native GlucDH had been determined to be about four by fluorescent titration using NADH (Pauly & Pfleiderer, 1975). In similar experiments no binding of NADH to the monomeric enzyme could be observed.

High concentrations of the oxidized coenzyme NAD completely inhibit dissociation. Figure 6 demonstrates that the addition of 20 mM NAD stops the dissociation process but promotes reassociation only to a very small extent. Therefore the percentage of residual activity obtained after the addition of NAD corresponds to the fraction of enzyme with unchanged coenzyme binding sites. Obviously the formation of the binary enzyme-NAD complex stabilizes the oligomeric structure but becomes kinetically unfavorable once dissociation is complete. This effect has been used to calculate the residual coenzyme binding capacity shown in Figure 7. The dependence of the stabilizing effect on the concentration of coenzyme represents

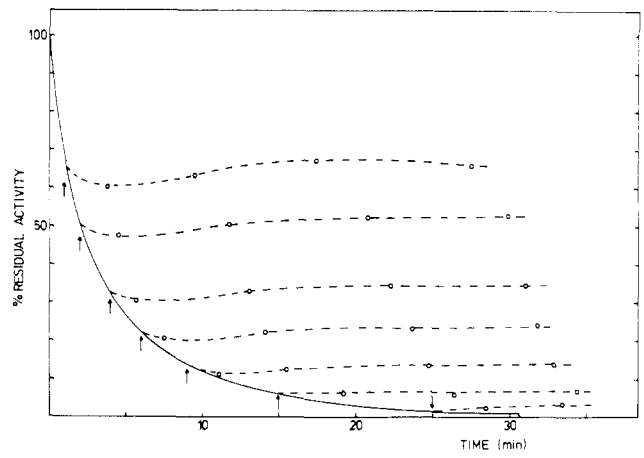

FIGURE 6: Influence of NAD on the dissociation of GlucDH. The decrease of activity of 5.2×10^{-7} M GlucDH caused by incubation in 50 mM Tris-HCl buffer (pH 8.5), containing 50 mM NaCl was recorded (—). At those times marked by arrows, aliquots were taken and adjusted to 20 mM NAD. Taking into account the dilution step the residual activity of these samples was followed.

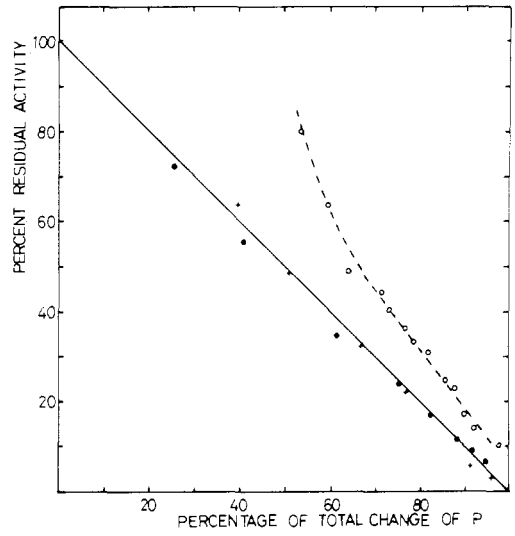

FIGURE 7: Percentage of residual activity vs. percentage of total changes of other spectroscopic and functional parameters during dissociation, such as: (○) polarization of intrinsic fluorescence at 290 nm; (+) NAD-binding capacity; (●) absorption at 292 nm.

TABLE II: Apparent Dissociation Constants of Binary GlucDH-Ligand Complexes.

Ligand	Dissociation constant (M)	G (kcal mol^{-1})
NAD	6×10^{-4}	−4.4
NADH	7.1×10^{-3}	−2.9
NADP	1.4×10^{-3}	−3.9
Adenosine 5′-diphosphoribose	4.9×10^{-3}	−3.1
Adenosine	4.4×10^{-3}	−3.2
Adenine	0.14	−1.2
AMP	5.5×10^{-2}	−1.72
ADP	5.2×10^{-2}	−1.75
Nicotinamide mononucleotide (NMN)		
AMP + NMN (1 + 1)	4.4×10^{-2}	−1.85
Ribose 5′-phosphate	6×10^{-2}	−1.7

a simple method to determine the apparent equilibrium constant of the enzyme–NAD complex. The dissociation constant 6×10^{-4} M for this complex shown in Table II agrees well with the value of 6.9×10^{-4} M earlier determined from initial rate measurements (Pauly & Pfleiderer, 1975). In order to evaluate which part of the coenzyme molecule is necessary for this stabilizing effect, various fragments of NAD were studied. The apparent constants of the corresponding binary enzyme–ligand complexes are summarized in Table II.

Correlation of Conformational and Functional Differences between Tetrameric and Monomeric Glucose Dehydrogenase. The change of conformational and functional parameters during dissociation was investigated in relationship to the change of enzymatic activity. Those parameters were the ability to form the enzyme–NAD complex, the absorbance at 292 nm, and the polarization of fluorescence at 290 nm.

The coenzyme binding capacity, the UV absorption, and enzymatic activity correlate precisely (Figure 6). No correlation, however, can be found between these parameters and the decrease of the polarization of fluorescence. A quick drop of the polarization to less than half of the total decrease is observed immediately after changing the pH from 6.5 to 8.5, while enzymatic activity still reaches 80% of the original value. The deprotonation of ionizable surfacial groups quickly renders more flexibility to the oligomeric structure without influencing the catalytic function. Dissociation and concomitant to it the other spectroscopic and functional changes of the enzyme follow much slower.

Discussion

The reversible dissociation and denaturation of GlucDH are impressive examples that under suitable environmental conditions the native configuration of an oligomeric enzyme is a result of its primary structure information (Anfinsen & Scheraga, 1975). Globular single polypeptide chains represent an intermediate state during the in vitro refolding of GlucDH and may be also obtained by dissociation of the native enzyme in weak alkaline solution. Dissociation of dehydrogenases so far investigated could only be achieved by conditions perturbating the conformation of the resulting protomers. Therefore information of limited value is available from the investigation of such monomeric species and reassociation often fails to yield conformers identical with the original state (Levi & Kaplan, 1971; Rudolph & Jaenicke, 1976).

The retention of the conformation of monomeric GlucDH monitored by optical rotatory dispersion and immunological reactivity indicates that subunit interaction seems to contribute only to a minor degree to the stabilization of the native folding of the single polypeptide chains. This may be one of the reasons why dissociation occurs under mild conditions and the subunits of GlucDH remain unchanged during at least 2 h after dissociation.

During the dissociation of various lactate dehydrogenases at pH 2.0, a similar monomeric state has been observed showing a half-life from 30 s to several minutes and full recovery of enzymatic activity when the enzymes were immediately returned to neutral conditions (Tenenbaum-Bayer & Levitzki, 1976; Vallee & Williams, 1975). When the time of exposure at low pH is increased, the fraction of activity recovery declines. Dissociated GludDH obviously corresponds to that short-lived intermediate stage during the acid dissociation of these enzymes.

Minor conformational changes, however, must take place as the monomers resulting from dissociation of GlucDH lack enzymatic activity. This fact can be explained by two different assumptions. In the first place dissociation may lead to a changed orientation of amino acid residues in the active site eliminating the coenzyme binding capacity. This assumption would imply that subunit interactions play a minor role for the retention of the gross tertiary structure but are essential to provide a catalytically active entity. In the second place inactivity of the protomers may be due to an increased segmental flexibility of the coenzyme binding site. This would result in a decrease of the affinity of this site for NAD without a change of the geometrical arrangement of functional groups involved in coenzyme binding. Such a transition from segmental flexibility to rigidity has been demonstrated to account for the acquisition of catalytic function in the system trypsinogen–trypsin (Huber et al., 1977) and has been postulated to induce cooperativity of hemoglobin (Abaturov et al., 1976). The low polarization of intrinsic fluorescence of the monomeric enzyme supports this second assumption. A shift in wavelength or change in relative strength of the electronic transitions in tryptophan absorption which are responsible for the UV-difference absorption spectrum may be expected to produce changes in the polarization spectrum only in the wavelength range covered by it (Anderson & Weber, 1965). The effect of various environments on the intrinsic fluorescence polarization spectrum of GlcDH is predominantly to produce a change of polarization over the whole wavelength range. Therefore these spectra mainly reflect changes in the local freedom of rotation of tryptophyl residues.

It has been known for a long time that the formation of the binary or ternary complex stabilizes oligomeric enzymes. Several authors demonstrated that this stabilization coincides with a tightening of the enzyme structure (DiSabato & Ottesen, 1965; Osborne & Hollaway, 1976). With regard to the binding constants reported for other dehydrogenases (McPherson, 1970; Osborne & Hallaway, 1976), only weak interaction is observed between GlucDH and AMP, ADP, or NADH, whereas adenine and adenosine are bound unusually tight. It may therefore be assumed that the stabilization of the tetramer originates from the binding of the adenine moiety of the coenzyme to GlucDH. This interaction of ligands with the adenosyl binding domain may inhibit the conformational fluctuations which are probably induced at higher pH values by an increased electrostatic repulsion of specific regions of the polypeptide chains. Kinetic and equilibrium studies concerning the influence of pH and specific ions on the reversible transition from the oligomeric to the well-defined monomeric state of GlucDH are in progress and will be soon reported.

References

Abaturov, L. V., Yakobashvily, N. N., Jinoria, K. Sh., Malchanova, T. P., & Varshavsky, Y. M. (1976) *FEBS Lett. 70,*

127-130.
Anderson, S., & Weber, G. (1966) *Arch. Biochem. Biophys. 116,* 207-223.
Anfinsen, C. B., & Scheraga, H. A. (1975) *Adv. Protein Chem. 29,* 205.
Banauch, D., Brünner, W., Ebeling, W., Metz, H., Rindfrey, G., Lang, H., Leybold, K., & Rick, W. (1975) *Z. Klin. Chem. Klin. Biochem. 3,* 101-107.
Chan, W. W.-C., & Mosbach, K. (1976) *Biochemistry 15,* 4215-4222.
Deal, W. C. (1969) *Biochemistry 8,* 2795-2805.
DiSabato, G., & Ottesen, M. (1965) *Biochemistry 4,* 422-428.
Engel, G. (1974) *Anal. Biochem. 61,* 184-191.
Hartmann, B. K., & Udenfried, S. (1969) *Anal. Biochem. 30,* 391-394.
Huber, R., Bode, W., Deisenhofer, J., Schwager, P., Fehlhammer, P., Colman, P., & Palm, W. (1977) *Hoppe-Seyler's Z. Physiol. Chem. 358,* 252.
Jaworek, D. (1969) *Chromatographia 2,* 289-292.
Levi, A. S., & Kaplan, N. O. (1971) *J. Biol. Chem. 246,* 6409-6417.
Mancini, G., Carbonara, A. O., & Heremans, J. F. (1965) *Immunochemistry 2,* 235-254.
Martin, R. G., & Ames, B. N. (1961) *J. Biol. Chem. 236,* 1372-1379.
McPherson, J. (1970) *J. Mol. Biol. 51,* 39-46.
Osborne, H. H. & Hollaway, M. R. (1976) *Biochem. J. 157,* 255-259.
Ouchterlony, Ö. (1948) *Ark. Kemi, Mineral. Geol. 268,* 1-9.
Pauly, H. E. & Pfleiderer, G. (1975) *Hoppe-Seyler's Z. Physiol. Chem. 356,* 1613-1623.
Rudolph, R., & Jaenicke, R. (1976) *Eur. J. Biochem. 63,* 409-417.
Sadoff, H. L., Bach, J. A., & Kools, J. W. (1965) in *Spores III* (Halvorson, H., Ed.) Ann Arbor Science Publishers, Ann Arbor, Mich., pp 97-110.
Tenenbaum-Bayer, H., & Levitzki, A. (1976) *Biochim. Biophys. Acta 445,* 261-279.
Vallee, R. B., & Williams, R. C. (1975) *Biochemistry 14,* 2574-2580.
Vimard, C., Orsini, G., & Goldberg, M. E. (1975) *Eur. J. Biochem. 51,* 521-527.
Weber, G., & Laurence, D. J. T. (1954) *Biochem. J. 56,* 31P.
Yanari, S., & Bovey, F. A. (1960) *J. Biol. Chem. 235,* 2818-2826.

Mechanism of the Irreversible Inhibition of γ-Aminobutyric Acid–α-Ketoglutaric Acid Transaminase by the Neurotoxin Gabaculine†

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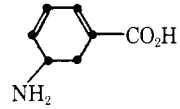
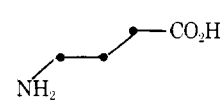
ABSTRACT: Gabaculine (5-amino-1,3-cyclohexadienylcarboxylic acid), a naturally occurring amino acid isolated from *Streptomyces toyocaenis,* is an irreversible inhibitor of bacterial pyridoxal phosphate linked γ-aminobutyric acid–α-ketoglutaric acid transaminase with a $t_{1/2}$ (25 °C) of 9 min at 3×10^{-7} M. Gabaculine is a substrate for γ-aminobutyric acid transaminase. The measured K_I is 2.86×10^{-6} M, and the k_{cat} for its turnover is 1.15×10^{-2} s^{-1} at 25 °C. When gabaculine is transaminated by the enzyme, it is converted to a cyclohexatrienyl system with one exo double bond. Upon spontaneous aromatization, this high energy intermediate is transformed into a stable *m*-anthranilic acid derivative (*m*-carboxyphenylpyridoxamine phosphate), which results in the covalent and irreversible modification of the cofactor. This adduct is bound tightly to the active site of the enzyme and can be liberated under denaturing conditions.

Naturally occurring irreversible enzyme inhibitors often function by a mechanism that requires their catalytic turnover by the target enzyme prior to the inhibition step (Rando, 1975). If a chemically reactive intermediate is generated, the inactivation of the enzyme can result from a reaction of this intermediate with an active-site residue or cofactor. Thus, in the process of catalytic turnover the enzyme becomes inactivated. The fact that these molecules are chemically unreactive before turnover is the key to their specificity. We would like to report here a further example of a naturally occurring irreversible inhibitor of this type. Specifically, we show that gabaculine (5-amino-1,3-cyclohexadienylcarboxylic acid), **1,** a natural



gabaculine



γ-aminobutyric acid

product isolated from *Streptomyces toyocaenis* (Mishima et al., 1976), is a potent irreversible inhibitor of bacterial, pyridoxal phosphate linked, γ-aminobutyric acid–α-ketoglutaric acid transaminase, and that the mechanism of action of this inhibitor involves its enzymatic transamination to form **2** followed by a subsequent aromatization step to generate *m*-carboxyphenylpyridoxamine phosphate (CPPp) (**3**). The net effect of this process is to covalently link the inhibitor to the cofactor. Although the mechanism of inactivation of γ-amino-

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