NCTC 945 cultures by Feder et al. (1971). Based on the zinc content, the molecular weight was thought to be 63 000, though the molecular weight was not determined. The zinc-free enzyme was inactive and Zn^{2+}, Co^{2+}, and Mn^{2+} restored 100, 230, and 26% of native activity, respectively. Its specific activity is twice that of the "microprotease" when the two are assayed under identical conditions. Such similarities minimally suggest a close relationship and optimally perhaps identity of the two proteins.

Thus, the *B. cereus* protease isolated from strain BRL-70 (Worthington Biochemical) is a monomeric protein of molecular weight 34 000, and there is no evidence of a polymeric structure. It is a zinc metalloenzyme with properties characteristic of bacterial neutral endoproteases. Hence, it is suggested that future reference to this protein should avoid the misnomer "microprotease." The convenient source and ease of purification of this protease described here provide an additional system to explore energy transfer measurements between metals at specific protein sites (Horrocks et al., 1975). Studies along these lines including Tb^{3+} incorporation and spectral studies of cobalt enzyme are in progress.

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Pulse Fluorimetry Study of Octopine Dehydrogenase–Reduced Nicotinamide Adenine Dinucleotide Complexes†

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ABSTRACT: We measured the transient fluorescence of NADH bound to octopine dehydrogenase in the binary octopine dehydrogenase–NADH complex and in the ternary complexes containing D-octopine, L-allooctopine, L-arginine, D-arginine, or 5-guanidinovaleric acid. The fluorescence decay in all these complexes is biexponential. This is explained by the presence of several conformations of the single NADH binding site. In addition, transient anisotropy measurements show that the nicotinamide moiety is rigidly bound to the enzyme.

Octopine dehydrogenase from muscles of *Pecten maximus* L. catalyzes reversibly the dehydrogenation of D-octopine to give L-arginine and pyruvate according to Scheme I (Thoai and Robin, 1961; Thoai et al., 1969). The enzyme is a monomer of molecular weight 38 000, with a single polypeptide chain and a single active site (Huc et al., 1971; Olomucki et al., 1972; Thomé-Beau and Olomucki, 1973).

The results of kinetic studies are consistent with a bi-ter sequential mechanism, in which NAD^+ [1] binds first to the enzyme followed by D-octopine and the products are released in the order L-arginine, pyruvate, and NADH (Doublet and Olomucki, 1975). The activity of ODH seems to be submitted to two types of regulation: a regulatory mechanism of memory

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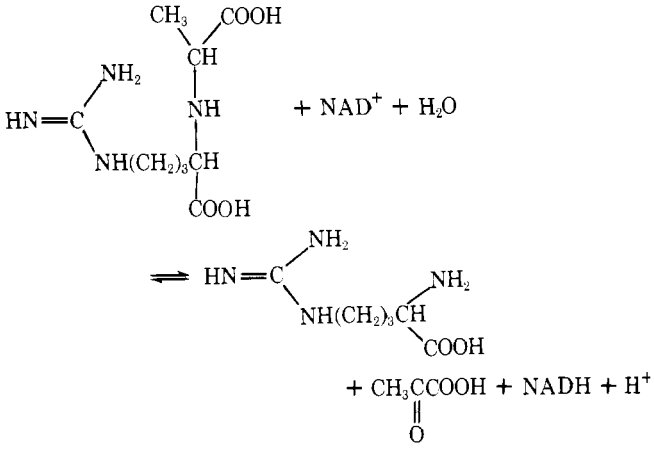
[1] Abbreviations used are: ODH, octopine dehydrogenase; GDH, beef liver glutamate dehydrogenase; LADH, liver alcohol dehydrogenase; TPB, 1,1,4,4-tetraphenyl-1,3-butadiene; DTT, dithiothreitol; NAD, nicotinamide adenine dinucleotide; NADH, reduced NAD; NADPH, NADH phosphate; EDTA, (ethylenedinitrilo)tetraacetic acid.

SCHEME I



type and the inhibitory effect of nonproductive complexes (Doublet et al., in preparation).

It was shown by spectroscopic studies using different analogues of the ligands that the enzyme is very specific for its substrates. The adenine and the pyridine parts are both involved in the binding of the coenzyme (Pho et al., 1970; Olomucki et al., 1975). The fluorescence intensity of the reduced coenzyme is strongly increased after the binding. This enhancement is greater in the ternary complex containing L-arginine or D-octopine than in the binary complex (Luisi et al., 1973; Baici et al., 1974). The fluorescence decays of NADPH-beef liver glutamate dehydrogenase and NADH-horse liver alcohol dehydrogenase have been recently measured (Brochon et al., 1976; Gafni and Brand, 1976). It was found that the NAD(P)H fluorescence decays did not follow a single exponential, but could be described by a sum of two exponentials. The fluorescence decays were independent of the coenzyme concentration, which implied that they were independent of the number of active sites occupied. Both enzymes studied were oligomeric with regulatory properties. It is therefore of interest to compare them with an enzyme like ODH, which is monomeric and has no allosteric properties.

In this work we used pulse fluorimetry to study the ODH-NADH binary complex and the ternary complexes obtained with the substrates L-arginine, D-octopine, and their analogues D-arginine, 5-guanidinovaleric acid, and L-allooctopine. All these complexes showed biexponential fluorescence decays.

Materials and Methods

Octopine dehydrogenase of *Pecten maximus* L. was purified and assayed as previously described (Thoai et al., 1969). The enzyme was stored at 4 °C as a suspension in ammonium sulfate. Before each series of experiments, the suspension was centrifuged and the pellet was dissolved in a minimum volume of buffer. The solution was dialyzed overnight against buffer and then clarified by centrifugation. The enzyme solution can be stored for several days at the concentration of 15 mg/mL and for 24 h at a dilution of about 5000 in the presence of 0.1 mM dithiothreitol.

D-Arginine and NADH were purchased from Sigma and L-arginine was from Calbiochem. L-Allooctopine was a gift from Dr. J. F. Biellmann (Biellmann et al., 1977) and D-octopine (isolated from the muscle of *Sepia officinalis*) from Dr. Y. Robin (Robin and Guillou, in preparation). 5-Guanidinovaleric acid was synthesized according to Schutte (1943). Fluorescence measurements were performed in a 0.05 M potassium phosphate buffer, pH 7.1, containing 0.1 mM EDTA and 0.1 mM dithiothreitol. The molar concentrations of NADH and enzyme solutions were determined spectrophotometrically at 340 (ϵ 6200 M^{-1} cm^{-1}) and 280 nm (ϵ 43 300 M^{-1} cm^{-1}), respectively.

Fluorescence Measurements. Transient fluorescence was measured by the photon-counting method (Wahl, 1969; Wahl and Auchet, 1972; Wahl, 1975). The photomultipliers used were a Radiotechnique 56 DUVP for polarization measurements and a RCA 8850 for the whole NADH fluorescence. The excitation light was provided by a high-pressure N_2 flash lamp. The excitation wavelength was selected by a M.T.O. interference filter centered at 336 nm ($\Delta\lambda$ 4 nm). The emission was collected through an interference filter M.T.O. centered at 466 nm ($\Delta\lambda$ 20 nm) associated with three cut-down filters: Schott GG 395, M.T.O. J 377 a, J 385 a. In some measurements the excitation and emission wavelengths were selected by 250- and 500-nm Bausch & Lomb monochromators, respectively. The flash frequency was about 15 kHz. Other details of the experimental procedure were as previously described (Brochon et al., 1976). The emission fluorescence spectra were obtained with a Jobin-Yvon spectrofluorimeter modified in the laboratory. They were corrected according to the wavelength dependence of the photomultiplier quantum efficiency and to the dispersion of the prism monochromator.

Transient Fluorescence Analysis. The experimental transient fluorescences can be considered as convolution products such as:

$$i(t) = \int_0^t g(T)I(t - T)\,dT \qquad (1)$$

where $I(t)$ is the fluorescence decay. The apparatus response function $g(t)$ was obtained by using a reference compound according to the method described elsewhere (Wahl et al., 1974). In the present work the reference compound was 1,1,4,4-tetraphenyl-1,3-butadiene in deaerated cyclohexane (see Brochon et al., 1976). The fluorescence decays $I(t)$ were assumed to be sums of exponential functions:

$$I(t) = \sum_{i=1}^{p} A_i \exp(-t/\tau_i) \qquad (2)$$

The τ_i values were the decay times and A_i values the amplitudes of the exponential terms. In the following tables of results, we used the relative amplitude defined as:

$$C_i = A_i / \sum_{i=1}^{p} A_i \qquad (3)$$

with

$$\sum C_i = 1 \qquad (4)$$

We determined the parameters τ_i and C_i which gave the best fit between the convolution product curve calculated from eq 1 and the experimental curve. The analysis of transient fluorescence was described in our previous work on pulse fluorimetry study of GDH-NADPH complexes (Brochon et al., 1976). We checked the correctness of the decay parameters by examining the deviation function $DV(t)$ and the value of the weighted residual R.

Analysis of the Transient Polarized Fluorescence. The two principal polarized components of the transient fluorescence were measured according to a method already described (Wahl, 1975). The curves $s(t) = i_{\parallel}(t) + 2i_{\perp}(t)$ were then deconvoluted and the corresponding decay $S(t)$ was determined. We finally analyzed the curves $d(t) = i_{\parallel}(t) - i_{\perp}(t)$, assuming the decay $D(t)$ to be:

$$D(t) = r(t)S(t) \qquad (5)$$

TABLE I: Fluorescence Decay Parameters of ODH-NADH Binary and Ternary Complexes.[a]

	pH	Temp (°C)	ODH (μM)	NADH (μM)	τ_1 (ns)	τ_2 (ns)	C_1	$\langle\tau\rangle$ (ns)	Residual
ODH-NADH	7	10	100	6.7	1.2	3.1	0.46	2.18	1.8
ODH-NADH-D-octopine[b]	6[d]	10	42	2.7	2.6	7.6	0.39	5.66	1.6
	7	10	40	5	1.75	7.0	0.38	5.01	3.2
	7	10	40	10	2.4	7.1	0.36	5.40	2.4
	7	10	40	20	1.6	6.5	0.40	4.54	2.8
	7	21	40	15	1.8	6.2	0.55	3.78	3.3
	8.8[e]	10	14.6	2.1	1.9	7.1	0.44	4.80	1.4
ODH-NADH-allooctopine[c]	7[d]	10	45	7	3.0	9.2	0.36	6.96	2.7
ODH-NADH-L-arginine	7	3	40	10	2.4	5.5	0.68	3.39	2.3
	7	10	40	10	2.05	5.6	0.74	2.97	3.2
	7	21	40	10	2.3	5.8	0.78	2.89	2.8
	7	21	40	15	2.1	5.7	0.78	2.89	2.7
	7	30	40	10	1.95	4.95	0.72	2.79	2.3
	8.8[e]	10	16.7	2.4	2.25	6.0	0.95	2.43	1.5
ODH-NADH-D-arginine	7	10	40	10	1.1	3.45	0.59	2.06	2.0
ODH-NADH-5-guanidinovaleric acid	7	10	40	10	1.9	4.1	0.91	2.1	2.5

[a] Experimental conditions were 0.05 M potassium phosphate buffer, 0.1 mM EDTA, 0.1 mM dithiothreitol. The concentration of the second ligand was generally 20 mM. The wavelengths of excitation and emission were selected by interference filters centered at λ_{exc} 336 nm and λ_{em} 466 nm, respectively. [b] D-Octopine concentration = 85 mM. [c] Allooctopine concentration = 75 mM. [d] $\Delta\lambda_{exc}$ 10 nm, $\Delta\lambda_{em}$ 11 nm (excitation and emission monochromators with λ_{exc} 336 nm and λ_{em} 440 nm). [e] $\Delta\lambda_{exc}$ 7 nm and $\Delta\lambda_{em}$ 9.5 nm, respectively (excitation and emission monochromators with λ_{exc} 336 nm and λ_{em} 440 nm).

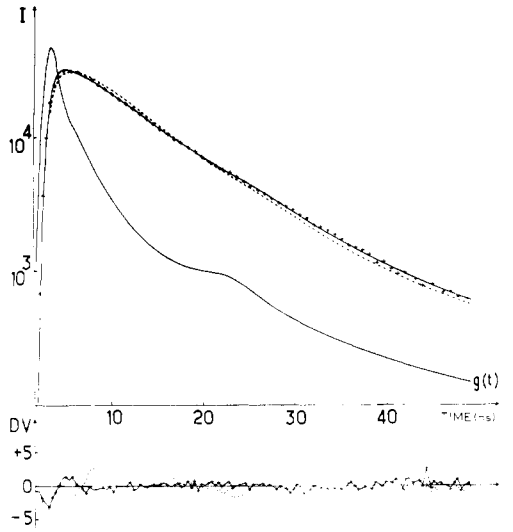


FIGURE 1: Transient fluorescence of NADH bound to ODH-NADH-D-octopine complex. Upper curves: experimental points (xxx), calculated convolutions with the best single exponential function (- - -) and the best biexponential function (—). $g(t)$ is the apparatus response function. Lower curves: deviation functions for the single (- - -) and the biexponential decays (—).

in which the fluorescence anisotropy $r(t)$ was a single exponential function (Jablonski, 1961)

$$r(t) = r_0 \exp(-t/\theta) \tag{6}$$

where θ is the correlation time defined by:

$$\theta = \frac{\eta V}{kT} \tag{7}$$

η is the viscosity of the solvent at the absolute temperature T, k is the Boltzmann constant, and V is the volume of the sphere equivalent to the molecule. We tested our analysis with the following deviation function and residual which are appropriate to the d(t) curve:

$$DV^k = \frac{d_c{}^k - d_{ex}{}^k}{i_\parallel{}^k + i_\perp{}^k} \tag{8}$$

$$R = \frac{1}{N}\sum_{i=1}^{N} (DV^k)^2$$

where $d_{ex}{}^k$ is the value of the experimental curve in the k^{ith} channel and $d_c{}^k$ is the value of the convolution product of $D(t)$ by the apparatus response function $g(t)$.

Results

Transient Fluorescence of NADH Bound to ODH in Various Complexes. We successively examined the binary complex ODH-NADH and the ternary complexes containing D-octopine, L-arginine, D-arginine, and 5-guanidinovaleric acid, respectively. The experimental conditions were chosen in order to obtain a saturation by the third ligand.

The transient fluorescence of the binary and the ternary complexes could not be satisfactorily reproduced by a single exponential: the best deviation functions $DV(t)$ diverged systematically from the time axis, along the whole experimental curves. With a sum of two exponential decays, however, it was possible to obtain a deviation function oscillating randomly on both sides of the time axis, as shown, for example, on Figures 1 and 2 which represent results obtained with the two ternary complexes ODH-NADH-D-octopine and ODH-NADH-L-arginine.

Each complex led to different values of decay parameters (Table I). No significant variation of the decay of D-octopine and L-arginine ternary complexes was observed with the coenzyme concentrations ranging from 5 to 15 μM. Moreover, the variation of temperature does not seem to affect significantly the value of C_1 ranging from 3 to 30 °C as shown in the case of the L-arginine ternary complex (Table I). We also examined the effect of pH on the D-octopine and L-arginine complexes.

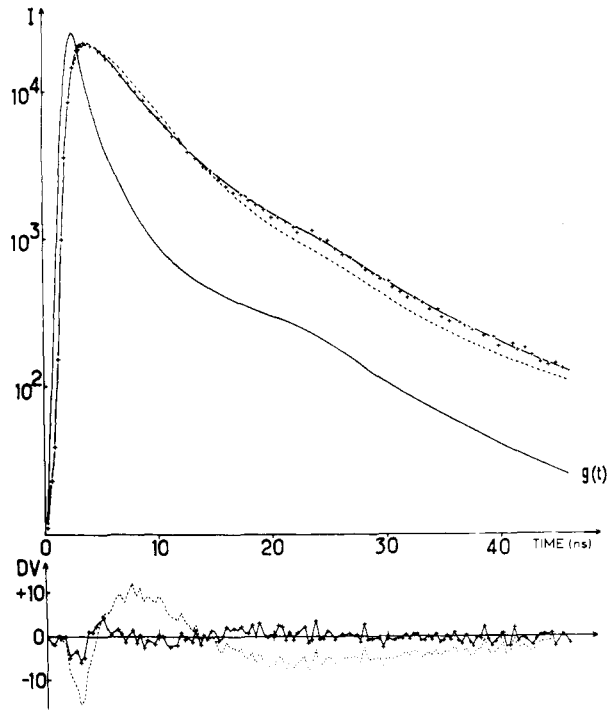


FIGURE 2: Transient fluorescence of NADH bound to the ODH-NADH-L-arginine complex. The meanings of the curve drawings are the same as in Figure 1.

We performed transient fluorescence measurements of the ODH-NADH-D-octopine complex at different emission and excitation wavelengths, and gathered the results in Table II, in which we can see no significant change in the decay parameters with emission or excitation wavelengths. No significant difference was found either between 440-nm emission and 550-nm emission with the allooctopine complex (not reported herein).

Fluorescence Emission Spectra of NADH in Different Complexes. We measured the fluorescence spectra of free NADH and of ODH-bound NADH in three ternary complexes where the second ligand was alternately 5-guanidinovaleric acid, L-arginine, and D-octopine. The excitation wavelength was 366 nm. These spectra are shown in Figure 3. A comparison of the results obtained with the different complexes shows that the increasing of the decay mean lifetime (Table I) is concomitant with the increasing blue shift of the emission spectra (Figure 3).

Fluorescence Anisotropy Decay of ODH-Bound NADH. We measured the polarized transient fluorescence of NADH in the two ternary complexes ODH-NADH-D-octopine and ODH-NADH-L-arginine. Figure 4 shows an example of the curves $s(t)$ and $d(t)$ for the L-arginine complex. The method of analysis described above shows that $r(t)$ is a single exponential. The values of correlation times θ and of the initial anisotropy r_0 are presented in Table III. From the enzyme molecular weight (38 000) and its partial specific volume (0.74 $cm^3\ g^{-1}$) (Olomucki et al., 1972) a dry volume of $V_D = 46.7$ nm^3 was calculated. Using eq 7, we obtained a correlation time $\theta_D = 15.6$ ns at 10 °C, which, compared to the measured correlation time, is about two times greater. Similar results are generally found with globular proteins and have been attributed to the hydration of the molecule (Yguerabide et al., 1970). The value of the initial fluorescence anisotropy, r_0, is close to the theoretical maximum value (0.4). The r_0 value of the D-octopine complex is greater than that of the L-arginine complex. This result might be related to the affinity of NADH to

TABLE II: Fluorescence Decay Parameters of the Ternary Complex ODH-NADH-D-Octopine as a Function of Excitation and Emission Wavelengths.[a]

λ_{exc} (nm)	λ_{em} (nm)	$\langle\tau\rangle$ (ns)	τ_1 (ns)	τ_2 (ns)	C_1	Residual
336	400	5.21	1.65	7.2	0.359	1.6
336	420	5.21	1.80	7.3	0.380	1.9
336	440	4.82	1.30	7.0	0.382	1.1
336	460	5.24	1.95	7.2	0.374	1.3
336	500	5.07	1.95	7.3	0.416	1.1
317	500	5.07	2.0	7.3	0.421	1.4
317	440	4.87	1.6	7.1	0.406	1.3
358	440	5.17	1.3	7.0	0.321	1.1
358	440	5.13	1.3	7.3	0.362	1.05

[a] Temperature was 10 °C. The bandwidths were 10 and 11 nm for excitations and emissions, respectively. Enzyme concentration was 5 μM. Other experimental conditions are given in the legend of Table I.

TABLE III: Anisotropy Decay Parameters of the Enzyme Bound NADH.[a]

Complexes	θ (ns)	r_0	Residual
ODH-NADH-L-arginine	33.5	0.329	1.2
ODH-NADH-D-octopine	33	0.361	1.1

[a] λ_{exc} 336 nm, λ_{em} 466 nm, t 10 °C, pH 7. The residual is obtained in the analysis of $D(t)$ curves.

the complexes, which is greater in the case of ODH-NADH-D-octopine than in the case of ODH-NADH-L-arginine (Luisi et al., 1973). The large values of r_0 indicate the absence of mobility of the dihydronicotinamide moiety of the coenzyme-NADH in the binding site.

Discussion

The data presented here indicate clearly that fluorescence decays of the reduced coenzyme bound to ODH are not single exponential functions. In order to explain these results, one is led to assume that the dihydronicotinamide moiety bound to ODH has a heterogeneous environment in its excited state. The question is whether this heterogeneity is preexisting in the ground state or not. With our present results, it does not seem possible to give a definite answer to that question.

If there was no heterogeneity in the ground state, some changes in the chromophore environment would occur at a rate comparable to the deexcitation rates, that is of the order of 10^9 s^{-1}. Much shorter rates would lead to single exponential decays, while much longer rates would not permit the changes to occur during the excited state (Donzel et al., 1974). The high values of the anisotropy r_0 at time zero and of the correlation θ indicate that the dihydronicotinamide moiety remains rigidly linked to the protein frame during the excited-states time. Then changes of the environment during the excited states should come from the motion of other chemical groups situated in the vicinity of the nicotinamide moiety. Such groups might be neighboring side chains of the protein.

The decay parameters were found to be independent of the excitation or emission wavelength. This result could mean that the difference of the interaction energies in the various environments is the same in the ground and excited states. This could also mean that these differences are small.

In order to explain a biexponential decay of the LADH-bound NADH, Gafni and Brand (1976) proposed the existence

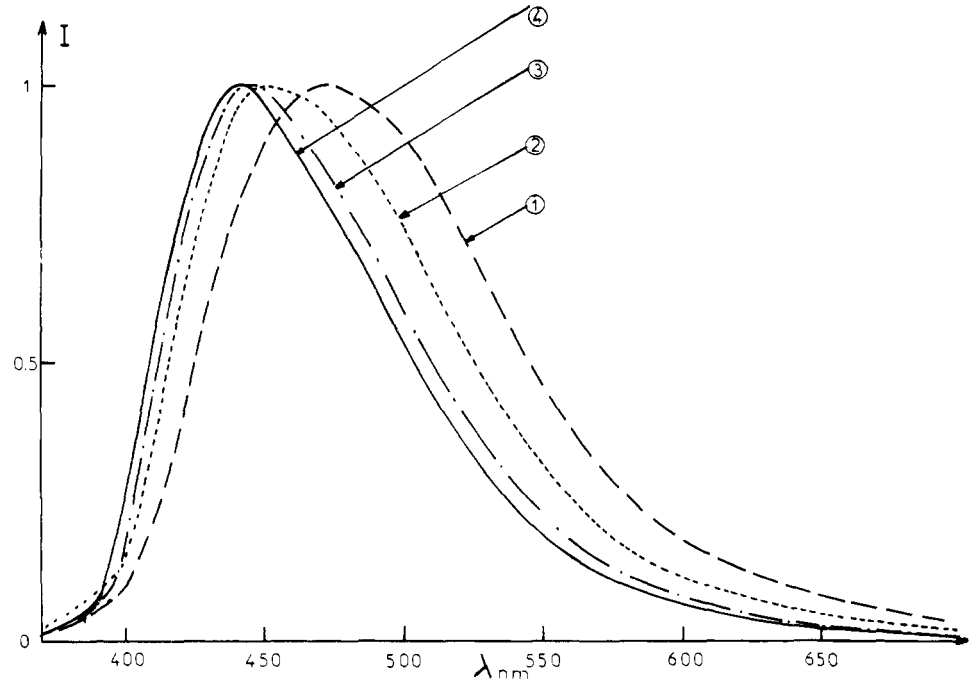


FIGURE 3: Fluorescence corrected spectra of NADH bound to ODH complexes: 1, free NADH; 2, ODH-NADH; 3, ODH-NADH-L-arginine; 4, ODH-NADH-D-octopine.

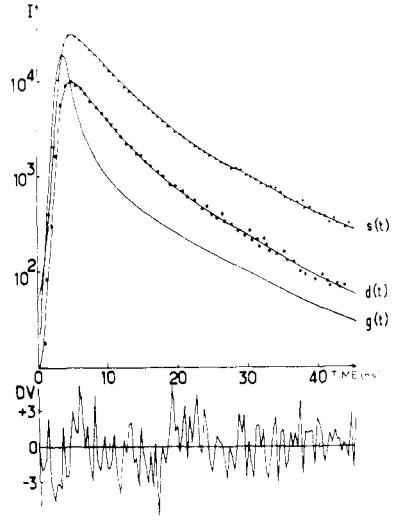


FIGURE 4: Transient fluorescence in polarized light of NADH bound to ODH-NADH-L-arginine complex. The crosses and points represent experimental values. The continuous curves are calculated convolutions. The lower curve represents the deviation function of the d(t) curve calculated according to eq 8.

of a reversible excited-state reaction which would transform the fluorescent chromophore to a nonfluorescent product. In order to be nonfluorescent, this product should have a very fast rate constant of deactivation, and, consequently, the rate of exchange in the excited state between the fluorescent and the nonfluorescent forms would be very great compared to the deactivation rate of the fluorescent product. Such a mechanism cannot lead by itself to a biexponential decay.

Let us examine some possible causes of environment heterogeneity of the nicotinamide ring in the ground state. The pK of the α-amino groups of the ligands such as D-octopine and L-arginine are 8.8 and 8.9, respectively. Then the protonated and nonprotonated forms are both present in the solutions studied. In the range of pH examined, the concentration ratios of these forms vary more than 10 times in the case of L-arginine and more than 100 times in the case of D-octopine. If the presence of the heterogeneous decay was due to the binding of both forms, a great variation of the amplitude C_1 would be expected in this range of pH, but that was not observed. The ionization of those groups would not be the factor influencing the environment of dihydronicotinamide when it is bound to the enzyme. Thus, the enzyme itself appears to create the heterogeneous environment of the bound coenzyme. The presence of multiple coenzyme sites cannot be observed on this enzyme as shown by gel filtration (Olomucki et al., 1972), polarimetry (Oriol and Olomucki, 1972), and steady-state fluorescence (Luisi et al., 1973; Baici et al., 1974). The most probable assumption is that there is some heterogeneity due to the existence of several conformations of the chemical groups surrounding the dihydronicotinamide.

It can be noticed that the variations with temperature of the decay parameters of NADH bound to ODH are small. This is in agreement with the temperature being practically independent of coenzyme affinities for the enzyme (Luisi et al., 1975).

Moreover, the blue shifts of the emission spectra of NADH in different complexes with ODH, related to the increasing of decay lifetimes, are concomitant to the tightness of NADH binding to the enzyme. This might indicate that the binding site of the coenzyme is hydrophobic. The hydrophobicity of the binding site is one of the features common to different dehydrogenases as was shown by recent x-ray studies (Ohlsson et al., 1974).

Biexponential fluorescence decays of NAD(P)H have been already found with the two other NAD(P)H dehydrogenases studied so far (Brochon et al., 1976; Gafni and Brand, 1976). Octopine dehydrogenase is a monomeric enzyme with a single active site which shows clearly that the origin of the multiexponential decays is due to the presence of several conformations of the site surrounding the dihydronicotinamide moiety, as it has already been postulated in the case of glutamate dehydrogenase (Brochon et al., 1976). But it seemed difficult so far to confirm that the situation really occurs during the catalysis, since it was dealt here with abortive complexes. Nevertheless,

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.

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

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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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