Conformation and Conformational Changes of Reduced Diphosphospyridine Nucleotide in Solution*

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ABSTRACT: The binding of reduced diphosphopyridine nucleotide to dehydrogenases can produce changes in its ultraviolet spectrum attributable to both the reduced nicotinamide and adenine moieties. Changes in coenzyme conformation have been proposed to explain the results of nuclear magnetic resonance studies on enzyme–coenzyme binding and fluorometric studies of the denaturation of reduced diphosphopyridine nucleotide in solution. Using solvent perturbation techniques, we described here the exposure of the coenzyme chromophores to solvent in the native conformation and after denaturation. These results, together with information obtained through the resolution of "denaturation" ultraviolet difference spectra into red or blue shifts, hyper- or hypochromicities, and the equivalent of an adenine pH difference spectrum, lead to the conclusion that the adenine ring is "stacked" to the nicotinamide ring, excluding water in the closed conformation of reduced diphosphopyridine nucleotide and reduced triphosphopyridine nucleotide in solution. We suggest that the use of "denaturation" difference spectra resolution components together with knowledge of coenzyme conformation will now facilitate interpretation of difference spectra arising from the binding of coenzyme to dehydrogenases.

It has been demonstrated that the binding of DPNH to specific pyridine nucleotide linked dehydrogenases causes changes in the 260- and 340-mμ absorption bands of the DPNH spectrum; and that, in some cases, these changes reflect binding at the active site of the enzyme (Theorell and Bonnichsen, 1951; Chance and Neilands, 1952; and Cross and Fisher, 1966).

Using fluorescence and fluorescence polarization studies, Velick (1958) proposed that different dehydrogenases bind DPNH in different conformations. Other studies using various techniques including nuclear magnetic resonance (Jardetzky and Wade-Jardetzky, 1966), ultraviolet absorption (Cilento and Schreier, 1964), fluorescence polarization (Churchich, 1967), and fluorescence relaxation (Czerlinski and Hommes, 1964) have suggested that DPNH can undergo conformational changes in solution and on binding to specific enzymes.

We have now found that conditions which change the conformation of the coenzyme in solution also cause changes in its ultraviolet spectrum; these changes have a certain general resemblance to those caused by enzyme binding.

The purpose of this paper, then, is to demonstrate and characterize ultraviolet difference signals arising from DPNH in order to elucidate the conformation of the coenzyme in solution. This information, applied to enzyme–coenzyme difference spectra, would assist in the determination of the conformation of the coenzyme on the enzyme surface and of changes in coenzyme conformation concomitant with enzyme–coenzyme complex formation.

Experimental Section

Materials. DPNH, Grade III, disodium salt, approximately 98%; TPNH, Type II, tetrasodium salt, 95–99%; reduced nicotinamide mononucleotide, Type III, sodium salt, approximately 90%; adenosine 5′-monophosphate (5′-AMP), Type III, sodium salt, 99–100%; and guanidine hydrochloride were all products of Sigma Chemical Corp., Saint Louis, Mo. Sucrose, mono- and dibasic potassium phosphate, phosphoric acid, urea, and ammonium chloride, all analytical reagent grade, were purchased from Mallinckrodt Chemical Works. Deuterium oxide, batch XXVIII, greater than 99.7%, was a product of General Dynamics Corp., Liquid Carbonic Division, San Carlos, Calif. Venom phosphodiesterase (lot no. 15) was purchased from Worthington Biochemical Corp., Freehold, N. J. Methanol (certified A.C.S. Grade) was a product of Fisher Scientific Co. The water used was charcoal filtered and doubly deionized.

All difference spectra were recorded on a Cary Model 14 double-beam spectrophotometer equipped with a deuterium light source and thermostated sample and reference cell holders regulated by Haake Model F thermostated baths at 20 ± .1°. The cells used were matched sets of fused quartz of 1.000-cm path length, quaracil grade, a product of Precision Cells, Inc. Absolute absorbance was read on a Zeiss PMQII spectrophotometer and refractive indices were measured on a ther-

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TABLE I: Determination of Adenine and Nicotinamide Exposure in Solutions of DPNH and TPNH.

Coenzyme or Analog	Perturbant	$(\Delta A/A)/\Delta n$ Reduced Nicotinamide (340 mμ)	$(\Delta A/A)/\Delta n$ Adenine (260 mμ)	% Exposure Reduced Nicotinamide	% Exposure Adenine
NMNH[a]	D_2O	8.99		100	
	Sucrose	*1.25*		*100*	
5′-AMP	D_2O		13.90		100
	Sucrose		*1.69*		*100*
DPNH	D_2O	6.72	11.43	75 ± 7	82 ± 7
	Sucrose	*0.81*	*1.01*	*65 ± 10*	*60 ± 7*
TPNH	D_2O	7.13	10.50	79 ± 8	76 ± 5
	Sucrose	*0.77*	*0.94*	*62 ± 6*	*56 ± 5*
DPNH in 12 M methanol	D_2O	8.75	14.18	97 ± 2	102 ± 3

[a] NMNH, reduced nicotinamide mononucleotide.

mostated Bausch & Lomb Abbe 3-L refractometer. A Radiometer Model PHM4c pH meter was used to measure all pH values. Curve resolution was accomplished using a DuPont Model 310 curve resolver equipped with an area integrator and recorder.

Methods

Difference Spectra. Solvent perturbation difference spectra were recorded using a tandem arrangement of cells in the sample and reference compartments in which the first cell in the sample compartment contained coenzyme or analog plus perturbant, and the second only buffer; while the first cell in the reference compartment contained coenzyme or analog in buffer, and the second contained the perturbant. To obtain the correct volumes and amounts of solutions and thus avoid dilution errors, the following procedure was used. All solutions to be pipetted were kept in a constant-temperature bath. Buffer (3 ml) or perturbant plus buffer were pipetted into the appropriate cells using the same pipet cleaned and dried between solution changes, and the base line was recorded on the spectrophotometer. Next the coenzyme or analog was added to the appropriate cells, using a single 50 μl of Lang–Levy pipet, and then stirred with nonwettable polypropylene stirrers. After a 10–15-min period to permit temperature equilibration and the settling of dust particles, the difference spectrum was recorded.

The same procedure used for solvent perturbation was also employed to obtain the operational or denaturation difference spectra using methanol, guanidine, or urea, and for the 5′-AMP pH difference spectrum. The difference spectrum of the splitting of coenzyme with venom phosphodiesterase was performed by addition of the enzyme to the cells after the base line was recorded with coenzyme solutions in the cells. A second addition of venom phosphodiesterase was made subsequent to the recording of a difference spectrum to assure completeness of the reaction. After recording the difference spectrum, the reference cuvet was used to measure A_{340} or A_{260}, and samples from the reference and sample cuvets were then used to determine the pH and refractive indices.

In order to avoid dilution errors (which appear as either hyper- or hypochromicities of all chromophores in the difference spectra) the pipets and vessels used must be scrupulously cleaned and dried. All solutions were filtered prior to use through a 0.22-μ Millipore filter to reduce the number of large particles which scatter light that contributes increasingly to the difference spectrum while scanning to lower wavelengths. Precise temperature control must be maintained as any differential between the sample and reference cells exhibits a solvent perturbation contribution to the difference spectrum due to the difference in refractive index.

The reversibility of the methanol–coenzyme difference spectrum was determined by using 2-ml samples in the cells, recording the difference spectrum, diluting all cells with one ml of buffer, and then recording the diminished difference spectrum.

Curve Resolution. Resolution of difference spectra was accomplished using a DuPont 310 curve resolver. The component models to be used were first resolved to the best fit using gaussian curves. The best fit was made in that spectral region contributing most to the difference spectrum to be resolved. Using these fits, the operational difference spectra were resolved by maintaining the predetermined ratio of areas of gaussian components to each model and varying the

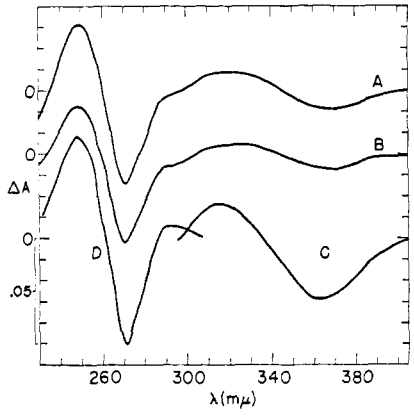

FIGURE 1: Solvent perturbation difference spectra of (A) DPNH, (B) TPNH, (C) reduced nicotinamide mononucleotide, and (D) 5′-adenosine monophosphate (5′-AMP); all 96.7 × 10^{-6} M in 95% (v/v) D_2O *vs.* the same in H_2O.

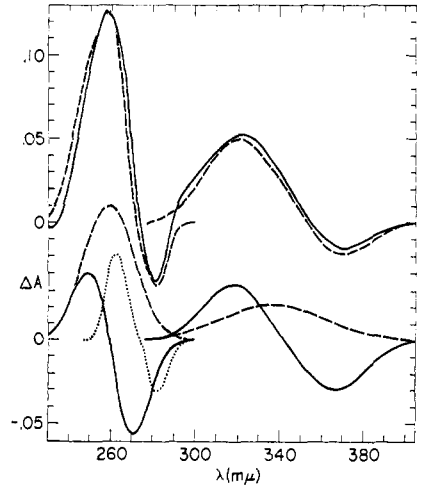

FIGURE 2: Denaturation difference spectrum of 96.7 × 10^{-6} M DPNH produced by using 3.2 μg/ml of venom phosphodiesterase (solid curve in upper portion of figure). The difference spectrum was resolved in to the curves shown in the lower portion of the figure. Hyper- or hypochromic components are the solid curves, red and blue shift components are dashed, and the pH component is dotted for both the reduced nicotinamide (340 mμ) and adenine (260 mμ) regions of the difference spectrum. The summation of these components is shown as the dashed line superimposed on the difference spectrum. Conditions are as described in the methods section and in the legend to Figure 9.

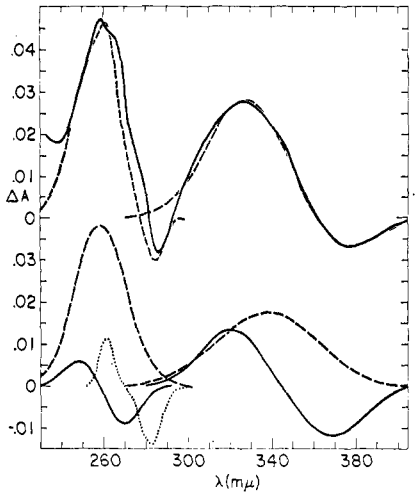

FIGURE 3: The difference spectrum of the denaturation of 96.7 × 10^{-6} M DPNH by 7.16 M methanol. The resolution of the difference spectrum and summation of the resolved components are as described in Figure 2.

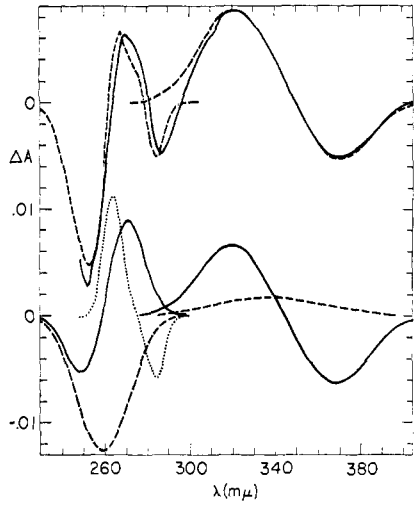

FIGURE 4: The difference spectrum of the denaturation of 98 × 10^{-6} M DPNH by 0.393 M guanidine. The resolution of the difference spectrum and summation of the resolved components are as described in Figure 2.

proportions and sense of one model to another to the best fit of the difference spectrum.[1]

Results

Solvent Perturbation Difference Spectra. Figure 1 shows typical perturbation spectra of DPNH, TPNH, and analogs of their components, reduced nicotinamide mononucleotide and 5′-AMP, by 94% (v/v) deuterium oxide. These difference spectra show blue shifts of the adenine and reduced nicotinamide absorption peaks. The perturbation of these chromophores by 0.73 M sucrose (diameter 9.74 Å) results in a red shift difference spectrum qualitatively identical with those in Figure 1 but opposite in sign. The dependence of coenzyme perturbation on perturber concentration was found to be linear through the concentration ranges of 24–94% (v/v) D_2O and 0.36–1.1 M sucrose. The dependence of perturbation upon coenzyme concentration was also linear from 25 to 148 μM DPNH and extrapolated to the ori-

[1] It should be noted that these resolutions are in no way dependent upon the use of the DuPont curve resolver. The same resolutions could be obtained by computer analysis or by hand plotting. The resolver provides fast and accurate solutions and thereby permits the rapid exploration of a large number of possible solutions and their quantitative variations.

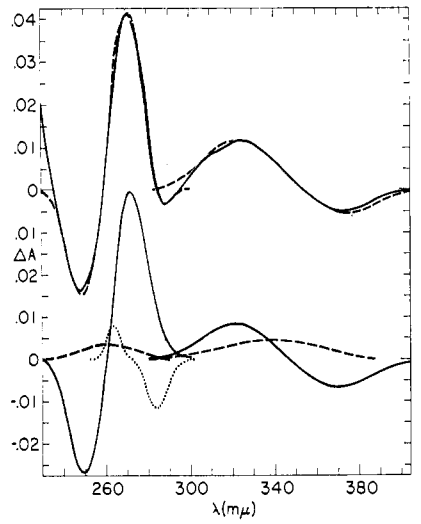

FIGURE 5: The difference spectrum of the denaturation of 98 $\times 10^{-6}$ M DPNH by 3.0 M urea. The resolution of the difference spectrum and summation of the resolved components are as described in Figure 2.

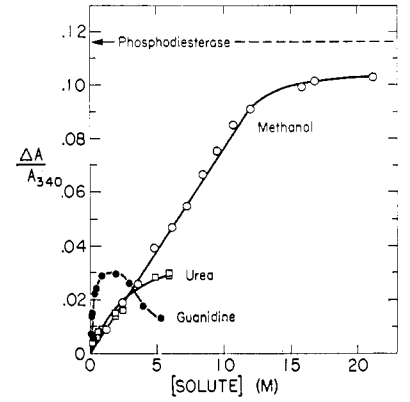

FIGURE 6: Dependence of the appearance of the reduced nicotinamide portion of the DPNH difference spectrum on concentration of denaturant. Methanol (○), guanidine (●), urea (□), and 3.2 μg/ml of venom phosphodiesterase.

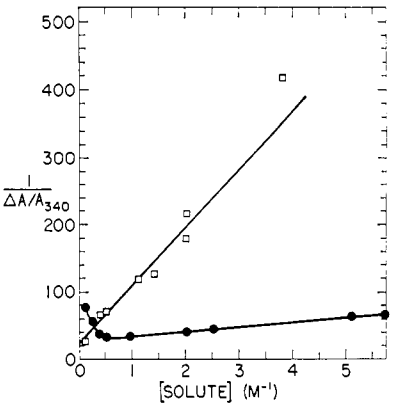

FIGURE 7: Double-reciprocal plots showing the binding dependence of the appearance of the reduced nicotinamide portion of the DPNH difference spectrum. K_d (guanidine) = 0.17 M and K_d (urea) = 3.3 M. The dependencies of the adenine portion of the DPNH difference spectra gave K_d's identical with those above.

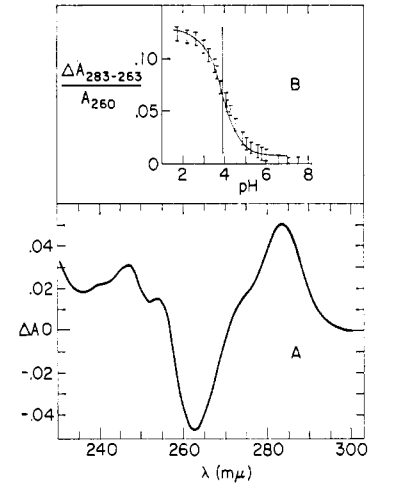

FIGURE 8: Adenosine pH difference spectrum. (A) The difference spectrum of 5′-adenosine monophosphate at pH 3.0 *vs.* the same at pH 7.6. (B) The pH dependence of the appearance of the difference spectrum ($\Delta A = \Delta A_{284}$ mμ $- \Delta A_{263}$ mμ) resulting in a pK of 3.9 and a $\Delta A/A$ (maximum) of 0.118. The pH titration was performed in 0.1 N KCl and 0.005 M potassium phosphate buffer. (——) Theoretical curve: pH = pK + log ΔA (max)/(ΔA − 1).

gin. Table I shows the results of the perturbation of DPNH and TPNH. The data are expressed as $(\Delta A/A)/\Delta n$ to normalize difference spectra obtained using different perturber and coenzyme concentrations, and represent averages of 5–13 individual difference spectra. ΔA values are measured as the difference in A between the trough and peak of the difference spectrum of each chromophore (A_{320}–A_{367} mμ) and (A_{249}–A_{271} mμ) for nicotinamide and adenine perturbation, respectively.

Operational Difference Spectra. The difference spectra of DPNH plus denaturant *vs.* DPNH alone are shown (solid curves in the upper portion of the figures) in Figures 2 to 5 for phosphodiesterase, methanol, guanidine, and urea.

The dependence of the total 340 mμ or nicotinamide portion of the difference spectrum upon solute concentration is shown in Figure 6. Figure 7 illustrates the binding dependence of the reduced nicotinamide difference spectra upon guanidine hydrochloride and urea concentration. The values from this plot are K_d (guanidine) = 0.17 M, K_d (urea) = 3.3 M; and $\Delta A/A_{340}$ (max) = 0.03–0.04 for both solutes.

A pH difference spectrum of 49.6 $\times 10^{-6}$ M 5′-AMP at pH 3.0 *vs.* pH 7.6 is shown in Figure 8A. Figure 8B is a plot of the reversible pH dependence of the appearance of the 260 mμ difference spectrum showing a pK of 3.9 and a $\Delta A/A$ maximum of 0.118.

Discussion

Solvent Perturbation. The exposure of reduced nicotinamide and adenine in DPNH relative to that of free nucleotide is measured using the solvent perturbation

method of Herskovits and Laskowski (1960). The inference is that some conformation of the coenzyme prevents intimate contact by the perturbant to these chromophores. Reduced nicotinamide mononucleotide and 5′-AMP[2] were chosen as models of 100% exposure because of their accessibility to the solvent.

Perturbation of DPNH by D_2O reveals that 25% of the nicotinamide residues and 18% of the adenine residues are buried to D_2O (and therefore, presumably to H_2O). These results can be interpreted in two ways: (1) that each molecule of DPNH has the above proportions of chromophores buried to water, or (2) that there is more than one conformer of coenzyme in solution in which the chromophores are buried in different proportions.

Jardetzky and Wade-Jardetzky (1966) using nuclear magnetic resonance and Czerlinski and Hommes (1964) using temperature-jump fluorescence concluded that there are two conformers of DPNH in solution and that they are in equilibrium with each other. A loss of fluorescence transfer was observed by Freed *et al.* (1967) in methanolic solutions of DPNH with no loss of the nicotinamide fluorescence. Table I shows that there is a complete exposure of the nicotinamide and adenine chromophores of DPNH to D_2O in 12 M methanol. This evidence permits us to conclude that there is one conformation of DPNH which buries chromophores to solvent more completely than in the open form.

The DPNH molecule is too small to bury completely both the adenine and nicotinamide residues in an intramolecular complex. Up to 50% of both chromophores could be buried if the rings are stacked so as to exclude water at their interfaces. If we assume that all of the chromophores buried to solvent reside in maximally closed coenzyme conformers and that in these conformers the chromophores are 50% buried to solvent, then we can estimate that those coenzyme chromophores which are shown in Table I to be 18–25% buried to water are thus buried in 40–50% of the coenzyme molecules in solution.

TPNH perturbed by D_2O shows essentially the same exposure as that of DPNH with the exception of the adenine moiety, which shows 6% less exposure; probably due to steric hindrance by the phosphate group on the C-2 atom of the adenylate ribose.

Both chromophores are 10–20% less accessible to sucrose (diameter, 9.74 Å) than to the smaller D_2O molecule, indicating that secondary structural details of the coenzyme hinder accessibility to the chromophores.

The linear dependence of the perturbation signal upon perturber concentration with extrapolation to the origin, ensures that the perturbants have not effected a change of conformation in the coenzyme in which chromophores have become either more buried or exposed. The relative perturbation ($\Delta A/A$) remains constant through the concentration range of DPNH used, confirming that intermolecular complexing is not involved.

Operational Difference Spectra. Whereas the results of solvent perturbation describe the accessibility of solvent to chromophores in the "native" conformation of DPNH, any operation which changes that environment through binding or conformational change will give rise to difference spectra which reflect the difference between the new or "denatured" environment and the "native" environment. Such difference spectra may result from differences in chromophore orientation or solvent accessibility to chromophores.

In the following sections we resolve operational difference spectra into either shifted or hyper–hypochromic difference spectral components. These resolutions are, of course, not mathematically unique as there are many other curves which would achieve an equivalent or perhaps better resolution. The justification of the choice of specific components depends upon three criteria: (1) the component must be demonstrated as an isolated observable phenomenon; (2) the system under consideration must be theoretically capable of undergoing changes which could produce such a component; and (3) a minimum number of such components should provide reasonably good resolutions of all of a great variety of difference spectra of a given chromophore.

It is now well established that any increase in the polarizability of the molecules immediately surrounding a chromophoric group causes a blue shift in the spectrum of that chromophore; a decrease in polarizability causes a red shift. This is precisely the phenomenon observed in solvent perturbation, where such difference spectra result from the difference in refractive indices between the sample and reference solutions. This phenomenon is generated by a wide variety of causes (Scheraga (1961) lists nine separate specific ones all related to polarizability). The shapes of the resulting difference spectra are quite independent of the specific phenomenon depending only upon the initial shape of the absorption band involved.

As has been noted by Chervenka (1959), and proven formally by Scheraga (1961), a difference spectrum produced by a simple shift of a spectral peak measured against the original peak has the form of the first derivative of the absorption band itself. While this is strictly true only for an infinitesimal shift, it is still an extremely good approximation for both solvent perturbation and coenzyme denaturation studies. These shifts involve small $\Delta\lambda$'s, on the order of 0.5–5.0 mμ, a range in which the resultant difference spectrum closely approximates the first derivative of the total spectrum. With these small shifts the height of the difference spectrum is proportional to $\Delta\lambda$ and therefore to Δn.

The alteration of a conformation of a molecule involving positive or negative changes in the refractive index of the immediate environment of chromophores would evidence red- and blue-shifted difference spectra, respectively. Aside from intramolecular interactions, the binding of a ligand to (or in the immediate vicinity of) a chromophore would produce relatively large red shifts as the local concentration of that ligand would be enormous.

Thus, observable shifts in the spectrum of a chromophore are attributable to either: (1) a change in general solvent environment, (2) a change in the specific environ-

[2] 5′-AMP has been reported to associate in solution but at concentrations several magnitudes higher than those employed in this study (Van Holde, 1967).

ment through conformational changes or ligand binding, or (3) a combination of both.

This causal generality predicts that the sine wavelike difference curve will be an important component of a difference spectrum caused by practically any interaction in which a chromophoric residue can conceivably participate.

Therefore we use a red- or blue-shifted solvent perturbation difference spectrum as the first component for resolution of the denatured coenzyme difference spectra.

The use of red- or blue-shifted difference spectrum components alone does not satisfactorily resolve the operational difference spectra. The remaining feature after subtraction of these red- or blue-shifted difference spectra resembled a spectrum of the chromophore itself. Therefore we choose hyper- or hypochromism as a second resolution component, especially since the requirements for this phenomenon are inherent in the DPNH molecule and hypochromism is a phenomenon common to polynucleotides. That is, there are transition dipoles of two planar rings which can themselves be stacked to exhibit hypochromism or aligned in a coplanar–colinear manner to exhibit hyperchromism. These effects are proportional changes in the extinction coefficient of the absorption band without a change in the wavelength of maximum absorption (Tinoco, 1960).[3] The resolution of the 340-mμ portion of the denaturation difference spectra in Figures 2–5 was then successfully achieved using the additional component of hyperchromism, a spectrum of reduced nicotinamide. This success suggests that the total absorption at 340 mμ exhibits, in this case, a general hyperchromism of all the electronic transitions involved in this absorption.

In attempting to resolve the 260-mμ (adenine) difference spectrum the use of any combination of shifted difference spectra plus hyper- or hypochromicity of the total 260-mμ absorption did not synthesize the 283–285-mμ trough present in all of the operational difference spectra. We were then forced to assume that this feature results from a modification of the absorption of a specific electronic transition. Two $\pi \rightarrow \pi^*$ and a single $n \rightarrow \pi^*$ transition have been ascribed to the 260-mμ absorption of adenine (Rich and Kasha, 1960; Voelter *et al.*, 1968). Rich and Kasha (1960) have demonstrated that on orientation of the two bases in polyadenylic and polyuridylic acids in a 1:1 helical complex (A + U), a hyperchromism occurs on the long-wavelength side of the 260-mμ absorption concomitant to a larger hypochromic effect at 260 mμ. They ascribe this hyperchromism to an orientation of the $n \rightarrow \pi^*$ transition. A difference spectrum synthesized from the data of Rich and Kasha shows a 283–285-mμ peak along with a larger 260-mμ trough. This feature resembles that found in the operational difference spectra as well as that seen in a pH difference spectrum of adenine[4] as shown in Figure 8A. The pK of the appearance of the pH difference spectrum was calculated from the data in Figure 8B and found to be 3.9 which approximates the value of 3.7 ascribed to the protonation[5] of the adenine ring at the N-1 position (Bock *et al.*, 1956; Zubay, 1958; Jardetzky *et al.*, 1963; Miles, 1961). This protonation would remove nonbonding electrons from the nitrogen and thus modify the absorption contribution of the $n \rightarrow \pi^*$ transition. The feature at 283–285 mμ then can be assumed to be a hypochromism as a result of the loss of nonbonding electrons. The use of this pH difference spectrum together with shifted difference spectra and hyper- or hypochromism does synthesize reasonable fits of the operational difference spectra.

The components, then, we use to identify environmental changes in the DPNH molecule are hypo- and hyperchromicities of the total absorption, blue and red shifts, and a pH difference spectrum of 5′-AMP.

Having identified the three components required for resolution of all of the DPNH denaturation difference spectra we have obtained, we now describe the specific resolutions of each difference spectrum and their interpretations.

Phosphodiesterase completely splits DPNH at the phosphate ester linkage into two components, 5′-AMP and NMNH, with a concomitant loss of fluorescence transfer between the adenine and nicotinamide moieties (Weber, 1957). The difference spectrum signals obtained by splitting coenzyme with phosphodiesterase, then, reflect only the intramolecular environment as compared to the environment of fully separated chromophores.

The difference spectrum of DPNH plus venom phosphodiesterase *vs.* DPNH shown in Figure 2 was resolved into the components shown in the lower part of Figure 2. The summation of these components is shown as the dashed line superimposed on the difference spectrum itself to show the fit. The resolution components and $\Delta A/A$ values are shown in Figure 9. A blue-shifted difference spectrum (D_2O perturbation of DPNH) was used to resolve both the nicotinamide and adenine portions of the difference spectrum and a spectrum of the 340- and 260-mμ contributions to a DPNH spectrum were used as hyperchromic contributions. The use of the above components in any proportion or sense did not give a satisfactory fit as the 283–285 mμ feature of the difference spectrum could not be synthesized. Therefore, a pH difference spectrum of 5′-AMP at pH 7.6 *vs.* 5′-AMP at pH 2.0 was used successfully as it contained the required feature.

[3] The literature frequently refers to any decrease in absorption measured at a single wavelength as a "hypochromicity." It is obvious that a red or blue shift will itself cause a decrease in absorbance over some range of the spectrum. We will reserve the terms "hypochromicity" and "hyperchromicity" for the phenomenon described by Tinoco as a change in absorbance without a shift of λ_{max}.

[4] Since the 283–285-mμ feature occurs in coenzyme solution in the *absence* of protein, caution should be exercised in attributing the occurrence of such features in enzyme–coenzyme difference spectra as being due to "protein conformational changes."

[5] Jardetzky and Jardetzky (1966), using PMR, demonstrated that the base protons of AMP in DPNH are slightly more shielded than free AMP thus demonstrating that there is no protonation of the adenine ring in free DPNH. We use the pH difference spectrum of AMP only as a model of possible hypochromicity of a component of the 260-mμ absorption band.

The enzymatic splitting of DPNH precludes any possibility of an intramolecular interaction between adenine and nicotinamide, thus the values of the resolved venom phosphodiesterase–DPNH difference spectrum in Figure 9 represent maximum values for the disorganization of the average conformation of DPNH in solution. If more than one DPNH conformer exists in solution then the denaturation of the more compact form would show a larger differential absorption than that of the more open form. Thus the A in $\Delta A/A$ represents the absorbance of all conformers in solution while ΔA is a function of the ratio of compact to open conformers.

Using the components required to resolve the venom phosphodiesterase–DPNH difference spectrum we can now describe the average initial environment of the chromophores in a solution of DPNH. The blue-shifted components of both the nicotinamide and adenine portions of the difference spectrum show that when the coenzyme is split these chromophores encounter an average environment of lower refractive index; thus they were partially buried to water in the native form as shown in the solvent perturbation section. The mutual hyperchromic contributions to the 260- and 340-mμ difference spectrum show that in the native form there was a coparallel, or stacked, orientation of the nicotinamide and adenine rings, which became disoriented when the coenzyme was split. The splitting of TPNH by venom phosphodiesterase produces difference spectra indistinguishable from those of the splitting of DPNH.

Freed *et al.* (1967) have shown that a loss of fluorescence transfer in DPNH and TPNH occurs in a methanolic solution and have proposed a conformational change to account for this loss. In the solvent perturbation section we showed that there is a subsequent exposure of both nicotinamide and adenine chromophores to water when methanol is present. Therefore, we obtained the methanol–DPNH operational difference spectrum shown in Figure 3 to determine those specific changes in the environment of adenine and nicotinamide which occur when DPNH is "denatured" by methanol.

The dependence of the differential absorbance of the 340-mμ region of the reduced nicotinamide difference spectrum upon methanol concentration is shown in Figure 6; it can be seen that on increasing methanol concentration, the magnitude of $\Delta A/A$ approaches the maximum as defined by the venom phosphodiesterase-split DPNH difference spectrum. The resolution of a typical methanol–DPNH difference spectrum is shown at the bottom of Figure 3 with the summation of the resolved components represented by the dashed line superimposed on the difference spectrum at the top of that figure.

It can be seen in Figure 9 that the components of this difference spectrum are the same as those found in the venom phosphodiesterase–DPNH difference spectrum; that is, blue-shifted components and hyperchromicities for both adenine and nicotinamide portions as well as an adenine pH contribution to the 283–285-mμ region. Because there is a significant concentration of methanol in the "denatured" DPNH solution, a red-shifted component from solvent perturbation is expected to contribute to the operational difference spectrum. However, the refractive index difference was small (on the order of 0.005 for a 6 M methanol solution), therefore the red shift contribution to the 340-mμ region only slightly diminishes the blue-shifted component of conformer opening. The methanol–DPNH difference spectrum appearance differs from that of venom phosphodiesterase–DPNH in that the proportionality of hyperchromicity to shift is not constant throughout the range of methanol concentrations and that there is a time-dependent appearance of 340- and 260-mμ hyperchromicity, especially at higher concentrations of methanol. These deviations from the venom phosphodiesterase-split difference spectrum possibly reflect the mechanism of the denaturation. We can say that methanol, like venom phosphodiesterase, causes a conformational change in the coenzyme in which both adenine and nicotinamide become disoriented from a stacked conformation and subsequently become exposed to the solvent. In addition, the adenine residues exhibit a similar difference spectrum contribution in the 283–285-mμ region of adenine absorption. Dilution of the methanol solution with water after recording the methanol–DPNH difference spectrum shows complete reversibility of the difference spectrum throughout the 2.0–6.5 M methanol concentration range.

It was of interest to determine if two common denaturants, guanidine and urea, would alter the conformation of DPNH in solution.

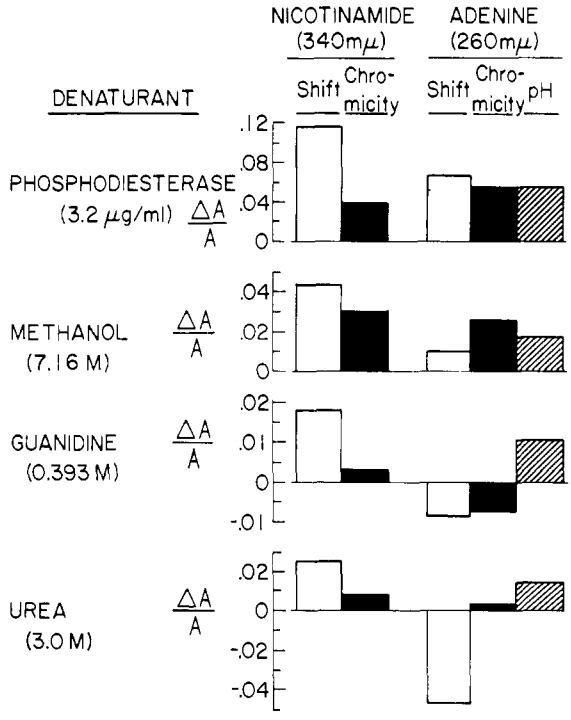

FIGURE 9: Comparison of the components required to resolve typical DPNH–denaturation difference spectra. Blue shift components are positive and red shift components are negative features of the graph. Hyper- and hypochromic contributions are represented as being positive and negative, respectively. The pH difference spectrum component of 5′-AMP at pH 7.6 *vs.* 5′-AMP at pH 3.0 is represented as a positive bar on the graph.

The difference spectra observed using these two denaturants (Figures 4 and 5) showed a similarity to the venom phosphodiesterase- or methanol–DPNH difference spectra in the 340-mμ region but both were markedly different in the 260-mμ portion of the difference spectra. The dependence of the 340 mμ signal on denaturant concentration is shown in the double-reciprocal plot in Figure 7. This dependence indicates that there was a specific interaction between coenzyme and solute, indicative of binding, with identical $\Delta A/A$ maxima and dissociation constants of 0.17 M guanidine and 3.3 M urea. The dependence of the 260-mμ (adenine) portion of the difference spectra gave dissociation constants identical with those of the 340-mμ region for both solutes. From the dependence of the 340-mμ difference spectrum on urea and guanidine concentration, as plotted in Figure 6, it can be seen that these values do not approach those obtained using either venom phosphodiesterase or methanol.

Figure 9 shows the amounts of the components used to resolve typical operational difference spectra of guanidine- and urea-denatured DPNH. The 340-mμ resolution for both denaturants includes both blue shift and hyperchromic components similar in proportion but of smaller magnitudes than those of the venom phosphodiesterase–DPNH data. Both denaturants show a 283–285 mμ or pH component of the adenine portion of the difference spectrum. Both difference spectra also show a red shift component in the 260-mμ region; urea shows a small hyperchromic contribution, whereas guanidine shows a hypochromic contribution to the 260-mμ adenine region.

Since the red shift component indicates that the adenine moiety encounters a general environment of higher refractive index than found in the average conformation of DPNH, and the nicotinamide blue shift component indicates that this chromophore is in an environment of lesser refractive index; it follows that the binding of the solutes on or near the adenine ring provides that high-refractive index environment by specific interaction with the coenzyme. Therefore, the binding dependence noted in Figure 7 is due to the binding of solute to, or very near, the adenine moiety of the coenzyme. It should be noted that the large refractive index contributions to the general solutions by urea and guanidine produce significant red shifts by solvent perturbation in both the nicotinamide and adenine portions of the difference spectrum, thereby diminishing the former and enhancing the latter. The novel hypochromicity of the adenine chromophore in the guanidine–DPNH difference spectrum may be a consequence of an orientation or interaction of the base and the guanidine chromophore. We then can say that both urea and guanidine do alter the native conformation of DPNH on binding at or near the adenine ring and that the nicotinamide–adenine orientation is disrupted in a manner such that the nicotinamide becomes more exposed to solvent. The magnitudes of the blue shift and hyperchromicity of the nicotinamide absorption do indicate that the denaturation process leads to a bound conformer of DPNH somewhat intermediate to the native form and that produced by methanol.

Summation

We can now generalize both our resolutions of operational difference spectra of DPNH and the conclusions to which these resolutions lead. (1) Under all denaturing conditions, the 340-mμ peak evidences a blue shift and a hyperchromicity while the 260-mμ absorption shows the 283–285-mμ feature seen in a pH difference spectrum of 5′-AMP. (2) In the absence of specific binding, the denaturation of coenzyme by venom phosphodiesterase or methanol shows difference spectra with blue shifts and hyperchromicities of the adenine absorption; while specific binding to the coenzyme by either urea or guanidine results in a red shift contribution to the adenine peak.

Determination of the "native" state of DPNH in solution by solvent perturbation, coupled with the resolutions of denaturation difference spectra we have just summarized, lead to the following specific conclusions. DPNH in aqueous solution exists as an equilibrium mixture of conformers in which 40–50% are closed. In this closed conformation the adenine and nicotinamide rings are mutually stacked excluding water at the ring interfaces. This conclusion is in substantial agreement with those reached by Miles and Urry (1968) using circular dichroism and by Sarma *et al.* (1968) using nuclear magnetic resonance. This closed conformation can be denatured nonspecifically by methanol with subsequent exposure of both adenine and nicotinamide chromophores to solvent, and specifically by binding to either guanidine or urea at the adenine moiety with partial exposure of the nicotinamide chromophore. Both denaturation processes evidence a disorientation of the mutually stacked conformation of nicotinamide and adenine chromophores.

We have already referred to the fact that the binding of DPNH to dehydrogenases generally produce ultraviolet difference spectra having features in the 340-, 280-, and 260-mμ regions. We have now found that our enzyme–coenzyme interaction difference spectra, while differing somewhat in detail from the DPNH denaturation difference spectra, are yet resolvable into diverse combinations of components some of which are identical with those used to achieve the resolutions reported here.

This work, relating changes in the spectrum of DPNH to changes in conformation of that coenzyme, may thus serve as a set of models to assist in the interpretation of dehydrogenase–coenzyme interaction spectra.

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The Isolation and Partial Characterization of Hydroxyproline-Rich Glycopeptides Obtained by Enzymic Degradation of Primary Cell Walls*

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With the Technical Assistance of Leon Clark

ABSTRACT: Enzymic degradation of cell walls isolated from suspension cultures of tomato released glycopeptides rich in hydroxyproline. Five glycopeptides accounted for about 20% of the total hydroxyproline, with approximate compositions as follows: (1) $Ara_{25}Gal_6Hyp_{10}Ser_3Tyr$, (2) $Ara_{14}Gal_3Hyp_{10}Ser_3Lys_2Thr,Val$, (3) $Ara_{20}Gal_4Hyp_9Ser_3Lys,Tyr$, (4) $Ara_{18}Gal_4Hyp_9Ser_3Tyr$, and (5) $Ara_{16}Gal_2Hyp_9Ser_3Lys_3Val,Tyr$. The composition and chemical properties of the glycopeptides indicated that the sugar amino acid linkage was a glycosidic link involving the hydroxyl group of hydroxyproline. This was confirmed by alkaline hydrolysis of the glycopeptides and subsequent isolation of hydroxyproline *O*-arabinosides.

In his classical work Heyn (1940) showed that the plant growth hormone auxin increases the plasticity of the primary cell wall and that plasticity is necessary for growth by cell extension.

The chemical basis for these changes in cell wall plasticity is unknown. Recently however I speculated (Lamport, 1965) that these changes might involve the hydroxyproline-rich cell wall protein, provisionally named "extensin," if it acted as a variable cross-link between wall polysaccharides. Two predictions followed: first the existence of a covalent linkage between extensin and the polysaccharides of the wall, and second the existence of *labile* cross-links directly affecting plasticity.

The data presented here verify the first prediction, after suitable enzymic degradation of cell walls we have been able to isolate hydroxproline-rich glycopeptides and identify the carbohydrate–protein linkage.

Our experimental approach involved a search for enzymes which would release hydroxyproline-rich material from isolated primary cell walls, the suitability of any degradation procedure being determined by the amount of peptide-bound hydroxyproline released and by its heterogeneity as judged initially from gel filtration on Sephadex columns. When choosing which cell walls to degrade we aimed for those richest in hydroxyproline and which also released the largest proportion of that hydroxyproline. We used cell suspension cultures because their use ensured the isolation of fairly pure preparations of primary cell walls rich in hydroxyproline.

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