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An Unusual Conformation of NAD^+ Bound to Sorbitol Dehydrogenase?

A Time-dependent Transferred Nuclear Overhauser Effect Study

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The conformation of NAD^+ in the sheep liver sorbitol dehydrogenase–NAD^+ binary complex has been investigated using time-dependent proton–proton transferred nuclear Overhauser enhancement measurements to determine interproton distance ratios and distances between bound NAD^+ protons. The conformation about both the adenosine and nicotinamide riboside glycosidic bonds is *anti*, the conformations of the adenosine and nicotinamide ribose rings are C3′-*endo* and C1′-*exo*, respectively, and the conformations about the adenosine and nicotinamide riboside C4′–C5′ bonds are g^+ and t, respectively, similar to those found in complexes of NAD^+ with other A type dehydrogenases. In addition, however, the distance data are indicative of an unusual overall conformation of NAD^+ in the sorbitol dehydrogenase–NAD^+ binary complex, with the planes of the nicotinamide and adenine rings separated by 6 to 8 Å and at approximately 120° to each other. This overall conformation differs from the concensus extended conformation found in the NAD^+–dehydrogenase complexes crystallized to date, where the planes of the nicotinamide and adenine rings are 12 to 14 Å apart and nearly perpendicular to each other.

1. Introduction

In α/β proteins, the loops linking the strands of β-sheet are connected in a right-handed manner, so, where there is a reversal of strand order, the connections lie on opposite sides of the sheet (Brändén, 1980). This arrangement generates crevices in which, in many cases, substrates or coenzymes bind (Brändén, 1980). Sites of this kind ($\beta\alpha\beta$ units) bind the coenzyme in dehydrogenases, and were among the first recurring supersecondary structures to be recognized (Ohlsson *et al.*, 1974; Rossmann *et al.*, 1974). A mononucleotide-binding fold, and a

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dinucleotide-binding fold were suggested (Rossmann *et al.*, 1977), and genetic relationships proposed (Rossmann *et al.*, 1975). These present-day structures were supposed to derive from a structural unit that was present in a very early ancestor, and developed into new enzymes throughout the history of life.

This type of $\beta\alpha\beta$ unit occurs in numerous and diverse proteins. They include adenylate kinase (Pai *et al.*, 1977), flavodoxin (Burnett *et al.*, 1974), glutathione reductase (Schulz *et al.*, 1978), *p*-hydroxybenzoate hydroxylase (Wierenga *et al.*, 1979), methionyl-tRNA synthetase (Zelwer *et al.*, 1982), phosphoglycerate kinase (Blake & Evans, 1974), phosphorylase (Weber *et al.*, 1978) and tyrosyl-tRNA synthetase (Bhat *et al.*, 1982). In all these enzymes, the $\beta\alpha\beta$ unit is involved in nucleotide binding, though with functional variations and uncertain genetic relationships. In human bladder p21 protein a $\beta\alpha\beta$ unit possibly binds GDP or GTP (cf. Gay & Walker, 1983; Wierenga & Hol, 1983). Replacement of Gly12 by Val (a guanine to thymine point mutation) in this protein produces a cancer-associated variant (Tabin *et al.*, 1982; Taparowsky *et al.*, 1982), possibly because this change alters nucleotide binding (Wierenga & Hol, 1983) and results in autophosphorylation at a threonine residue (Shih *et al.*, 1980). However, the three-dimensional structure is not known for either the normal p21 cellular protein or the homologous retroviral transforming gene product, and two interpretations of the Gly12 to Val replacement have been proposed, each based on a different alignment and model (Wierenga & Hol, 1983; Gay & Walker, 1983; Walker *et al.*, 1982).

A better knowledge of the structures and functions of $\beta\alpha\beta$ units is clearly necessary. Particularly interesting will be cases where the functions of the proteins are known, where evidence of genetic relationships between the proteins exists, and where the three-dimensional structure of at least one of the proteins is available at high resolution. Horse liver alcohol dehydrogenase (Eklund *et al.*, 1976), yeast alcohol dehydrogenase (Jörnvall, 1977*a*) and sheep liver sorbitol dehydrogenase (Jeffery *et al.*, 1981,1984) fulfil these criteria. In this paper, we report the use of a novel n.m.r. technique, namely time-dependent proton–proton transferred nuclear Overhauser enhancement measurements (Clore & Gronenborn, 1982*a*,1983), to determine interproton distance ratios and distances between

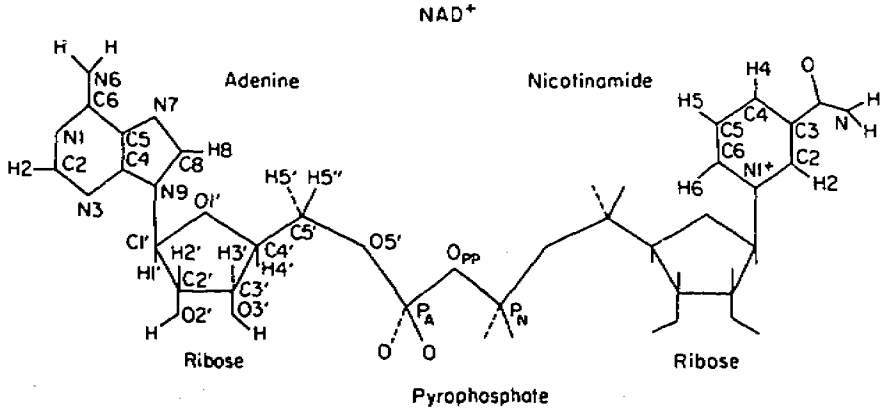

FIG. 1. Chemical structure of NAD^+. In the text protons of the adenosine and nicotinamide riboside moieties are distinguished by the subscripts A and N, respectively.

bound ligand protons for NAD$^+$ bound to SDHase†. We show that although the glycosidic bond and ribose conformations of both the adenosine and nicotinamide moieties of NAD$^+$ in the SDHase–NAD$^+$ binary complex are similar to those observed in complexes with the horse and yeast alcohol dehydrogenases (Gronenborn & Clore, 1982*a*; Clore & Gronenborn, 1983), the overall conformation of NAD$^+$ in the SDHase–NAD$^+$ complex appears to be different and unusual, folded though not stacked, with the planes of the nicotinamide and adenine rings separated by 6 to 8 Å and at approximately 120° to each other. This finding is somewhat surprising in the light of initial attempts to fit the amino acid sequence of SDHase to the crystallographically determined structure of horse liver alcohol dehydrogenase, which suggest a model for SDHase that appears to be not easily compatible with the presently deduced NAD$^+$ conformations (Eklund, H., Brändén, C.-I., Jörnvall, H. & Jeffery, J., unpublished data).

2. Materials and Methods

SDHase was purchased from Boehringer Mannheim GmbH and was 93% pure as judged by sodium dodecyl sulphate/polyacrylamide gel electrophoresis. After extensive dialysis against 50 mM-potassium phosphate, pH 7·2 (meter reading uncorrected for the isotope effect on the glass electrode) and 0·1 mM-dithioerythritol in D_2O, the solution was clarified by centrifugation and used without further purification. NAD$^+$ and 5′-AMP were obtained from Sigma Chemical Co. Ltd, lyophilized from D_2O, and used without further purification. All other chemicals used were of the highest purity commercially available. Samples for ^{1}H-n.m.r. contained 3·6 mM-nucleotide and 0·07 mM-SDHase (corresponding to 0·28 mM in nucleotide binding sites). (The concentration of SDHase was calculated on the basis of the SDHase subunit sequence, which consists of 354 amino acid residues and gives a calculated molecular weight of 37,837; the native enzyme has a tetrameric quaternary structure so that the molecular weight of native SDHase is 151,348; Jeffery *et al.*, 1984). Under these conditions the nucleotide binding sites are completely saturated with NAD$^+$ or 5′-AMP so that the ratio of free to bound nucleotide was 12. All experiments were carried out at 5°C. (Unfortunately, suitable conditions for carrying out n.m.r. experiments could not be obtained for nicotinamide mononucleotide as it was found to bind too weakly ($K_a < 100\ M^{-1}$) to SDHase.)

^{1}H n.m.r. measurements were carried out at 270 MHz using a Bruker WH-270 spectrometer. 480 transients were averaged for each spectrum using 4096 data points for a 4·2 kHz spectral width, and, prior to Fourier transformation, the free induction decay was multiplied by an exponential function equivalent to a line broadening of 2 Hz. The pulse sequence used in the time-dependent TRNOE experiments was $(t_1 - \pi/2 - AT - t_2)_n$ where the selective irradiation at a chosen frequency was applied during the time interval t_1 (0·002 to 0·8 s), AT is the acquisition time (0·487 s) and t_2 is a delay (4 s) to allow for complete recovery of magnetization of all protons to their equilibrium values prior to perturbation by the selective radiofrequency field. Chemical shifts are expressed relative to 2,2-dimethylsilapentane-5-sulphonate (used as an external standard).

The chemical structure of NAD$^+$ together with the nomenclature used to number the protons is shown in Fig. 1. The assignments of the base and sugar H1′ proton resonances of free NAD$^+$ were taken from Sarma & Mynott (1973). Homonuclear decoupling experiments were used to confirm these assignments and to obtain the assignments of the other sugar proton resonances. In the presence of SDHase, free and bound NAD$^+$ are in fast exchange on the chemical shift scale so that only a single set of averaged ligand

† Abbreviations used: SDHase, sheep liver sorbitol dehydrogenase (L-iditol:NAD$^+$ 5-oxidoreductase, EC 1.1.1.14); NOE, nuclear Overhauser effect; TRNOE, transferred nuclear Overhauser effect; n.m.r., nuclear magnetic resonance; p.p.m., parts per million.

resonances is seen at approximately the positions of the corresponding resonances of free NAD^+. Because of exchange broadening, certain groups of resonances are superimposed into a single averaged ligand resonance and can no longer be resolved individually, in particular the H_A2 and H_N5 resonances, the H_A1' and H_N1' resonances, the H_A3', H_N2' and H_N4' resonances, the H_A4', H_N3' and H_N5' resonances, and the H_A5', H_A5'' and H_N5'' resonances. In the time-dependent TRNOE measurements, the irradiation power was sufficient to be in the high power limit so that saturation can effectively be considered to be instantaneous, whilst preserving selectivity so that only a single averaged resonance at a time was saturated (Clore & Gronenborn, 1983). It should also be noted that the bandwidth of the applied radiofrequency field is $\sim 1/t$ (where t is the time for which it is applied), and the ratio of free to bound ligand of 12 was chosen so as to ensure that all initial slopes of the TRNOEs could be measured from irradiation times of $\gtrsim 50$ ms.

As an initial procedure we systematically irradiated the spectral region from 3·4 to 7·2 p.p.m. at 20 Hz (0·074 p.p.m.) intervals using a 0·4 s pre-saturation pulse. This region covers all the sugar resonances of NAD^+ and 5'-AMP and the α and β CH protons of the protein. In this procedure the selectivity of the TRNOEs is maintained since, in general, the extent of spin diffusion from indirect cross-relaxation *via* protons of the protein is approximately independent of the irradiation frequency providing this is placed within the protein resonance envelope (Clore & Gronenborn, 1983; and see for example Figure 5 of Clore & Gronenborn, 1982*a*, and Figure 1 of Clore & Gronenborn, 1982*b*). All the observed TRNOE effects on the base and H1' proton resonances were maximal and centred at the positions of the averaged ligand resonances strongly suggesting that these TRNOEs arise solely as a result of cross-relaxation between bound ligand protons. It should be noted, however, that in the absence of completely deuterated SDHase, it is impossible to exclude absolutely a direct TRNOE between a bound ligand proton and an α or β CH proton of the protein with a resonance position identical to that of one of the averaged ligand resonances. Such a possibility, however, is very unlikely on four counts: (1) the α and β CH resonances for a protein of $M_r \sim 150{,}000$ are broad ($\Delta\nu_{1/2} > 50$ Hz) and therefore difficult to saturate completely so that even with effective direct cross-relaxation between an α or β CH proton and a bound ligand proton a lag phase would be observed in the time development of the TRNOE; (2) one would not expect an α or β CH proton of the protein to be less than 4 Å from a bound ligand proton; (3) in our experience with other mono- and dinucleotide ligand-protein systems, including yeast and horse liver alcohol dehydrogenases (Gronenborn & Clore, 1982*a*), the cAMP receptor protein (Gronenborn & Clore, 1982*b*; Clore & Gronenborn, 1982*b*) and the Ca^{2+} ATPase in intact sarcoplasmic reticulum (Clore *et al.*, (1982), all TRNOEs on the base and H1' sugar resonances following irradiation within the sugar/α/β proton resonance envelope have always been maximal and centred at the positions of the averaged sugar resonances; and (4) the complete set of observed TRNOEs is self-consistent and compatible with a single conformation of the bound dinucleotide.

3. Results

The proton–proton NOE is a most powerful tool for structure determination in solution as it can be used to demonstrate the proximity of two protons in space and to determine their separation (Noggle & Schirmer, 1971; Redfield & Gupta, 1971; Poulsen *et al.*, 1980; Wagner *et al.*, 1981). The TRNOE involves the extension of NOE measurements to exchanging systems such as ligand–protein complexes, making use of chemical exchange between the free and bound states of the ligand to transfer magnetic information concerning cross-relaxation between bound ligand protons from the bound state to the free state (Clore & Gronenborn, 1982*a*,1983).

Figure 2 shows the 270 MHz ^1H n.m.r. spectrum of 3·6 mM-NAD^+ in the presence of 0·07 mM-SDHase corresponding to a ratio of free to bound NAD^+ of

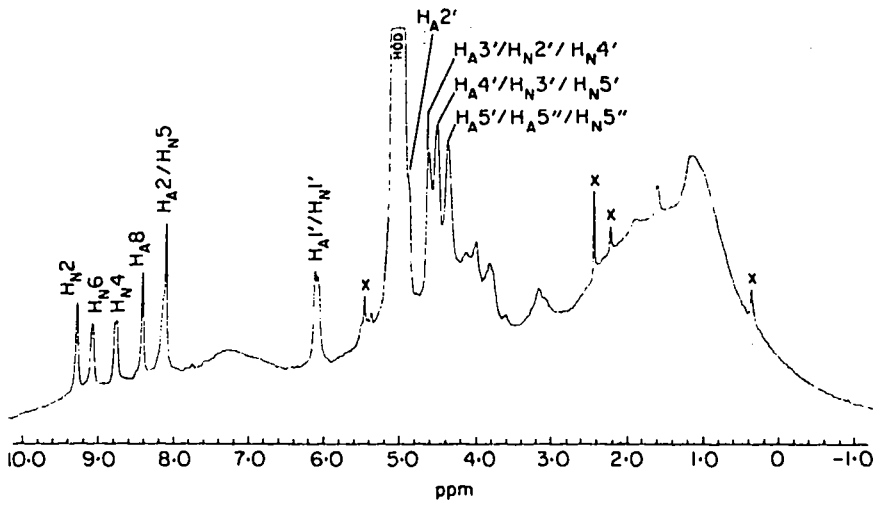

FIG. 2. 270 MHz ^{1}H n.m.r. spectrum of 3·6 mM-NAD$^+$ in the presence of 0·07 mM-SDHase corresponding to a ratio of free to bound NAD$^+$ of 12. Chemical exchange between free and bound NAD$^+$ is fast on the chemical shift scale so that only a single set of averaged exchange-broadened ligand resonances is seen at approximately the positions of the corresponding resonances of free NAD$^+$. The assignments of the averaged NAD$^+$ resonances are indicated. Other experimental conditions are given in the Materials and Methods section. (Peaks marked with an x are low molecular weight impurities.)

about 12, together with the assignments of the NAD$^+$ proton resonances. The binding of NAD$^+$ to SDHase is weak ($K \sim 5 \times 10^4$ M^{-1}; Christensen *et al.*, 1975) and chemical exchange between free and bound NAD$^+$ is fast on the chemical shift scale so that only a single set of averaged exchange-broadened ligand resonances is seen at approximately the positions of the corresponding resonances of free NAD$^+$. Thus, the TRNOE experiment involves irradiating each averaged ligand resonance in turn and observing the effect on the intensities of the other averaged ligand resonances. In a multiple spin system the initial slope of the time development of the TRNOE, $N_i(j)$, observed on the averaged ligand resonance of proton i following irradiation of the averaged ligand resonance j, is given by the weighted average of the cross-relaxation rates between protons i and j in the free and bound states (Clore & Gronenborn, 1983). In the case of NAD$^+$ free in solution, however, no NOEs could be observed between any proton pair for irradiation times <0·5 s so that the initial slopes of the TRNOEs observed in the NAD$^+$–SDHase system are simply given by:

$$\left.\frac{dN_i(j)}{dt}\right|_{t=0} = (1-a)\sigma_{i_B j_B} \tag{1}$$

where $\sigma_{i_B j_B}$ is the cross-relaxation rate between the bound ligand protons i_B and j_B, and a is the mole fraction of free ligand. Equation (1) is valid providing that in the case of the bound ligand protons, i_B and j_B, either

$$|\sigma_{i_B j_B}| \gtrsim |\sigma_{i_B l_B}|, \quad |\sigma_{i_B k_X}| \tag{2}$$

or

$$|\sigma_{i_B j_B}| \gtrsim |\sigma_{j_B l_B}|, \quad |\sigma_{j_B k_X}|, \tag{3}$$

where $\sigma_{i_B l_B}$ and $\sigma_{j_B l_B}$ are the cross-relaxation rates between a third bound ligand proton l_B and the bound ligand protons i_B and j_B, respectively, and $\sigma_{i_B k_X}$ and $\sigma_{j_B k_X}$ are the cross-relaxation rates between a protein proton k_X in the ligand–protein complex and the bound ligand protons i_B and j_B, respectively (Clore & Gronenborn, 1983). The two conditions (2) and (3) are sufficiently discriminating to ensure the validity of equation (1) for all time-dependent TRNOEs measured in this paper, as, in practice, to break both conditions would require the bound ligand proton l_B or the protein proton k_X to be less than ~ 2 Å from both bound ligand protons i_B and j_B. Using equation (1) cross-relaxation rates between pairs of bound ligand protons can therefore be determined directly and with ease, thus enabling distance ratios between any two such pairs of protons to be calculated, or, if one of the interproton distances is known, interproton distances to be calculated from the equation:

$$r_{i_B j_B}/r_{l_B m_B} = (\sigma_{l_B m_B}/\sigma_{i_B j_B})^{1/6} \tag{4}$$

(assuming a single correlation time for all the corresponding interproton distance vectors).

The complete time dependence of the TRNOE observed on an averaged ligand proton resonance i following irradiation of an averaged ligand proton resonance j is multiphasic, but for the purpose of description can be divided into two kinetic phases (Clore & Gronenborn, 1983). When the distance $r_{i_B j_B}$ is less than 4 Å, direct cross-relaxation between the two bound ligand protons i_B and j_B is an effective process and the initial phase is characterized by a decrease in the intensity of the averaged ligand proton resonance i from which the initial slope and, hence, the cross-relaxation rate $\sigma_{i_B j_B}$ can be determined. When $r_{i_B j_B}$ is greater than 4 Å, however, the initial phase is characterized by a lag owing to the fact that direct cross-relaxation between protons i_B and j_B is insignificant, and, consequently, $\sigma_{i_B j_B}$ cannot be determined. The second phase is characterized by a progressive decrease in intensity of the averaged ligand proton resonance i due to highly effective indirect cross-relaxation between many protons, a phenomenon known as spin diffusion.

The time dependence of the TRNOEs observed on the averaged H_A8, H_A2/H_N5, H_A1'/H_N1', H_N6, H_N4 and H_N2 resonances of NAD^+ following irradiation of the

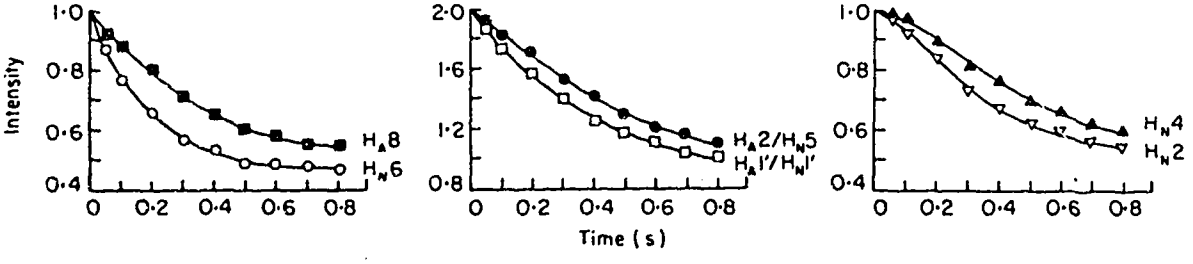

FIG. 3. Time dependence of the TRNOEs observed on the averaged H_A8, H_A2/H_N5, H_A1'/H_N1', H_N6, H_N4 and H_N2 resonances of NAD^+ following irradiation of the averaged $H_A3'/H_N2'/H_N4'$ resonance of NAD^+ in the presence of SDHase with a ratio of free to bound ligand of 12. The experimental conditions are as in the Fig. 2 legend.

averaged $H_A3'/H_N2'/H_N4'$ resonance of NAD$^+$ in the presence of SDHase with a ratio of free to bound ligand of 12 is shown in Figure 3. It can be clearly seen that the time-courses of the TRNOEs observed on the averaged H_A8, H_A1'/H_N1' and H_A2/H_N5 resonances are characterized by a rapid initial decrease in intensity indicative of effective and significant direct cross-relaxation, whereas those observed on the averaged H_N4 and H_N2 resonances are characterized by an initial lag phase indicating that direct cross-relaxation is insignificant. The complete set of observed initial rates for the time development of the TRNOEs in the NAD$^+$–SDHase system is collected in Table 1.

Although there are both overlap and superposition of the sugar resonances of the adenosine and nicotinamide riboside moieties of NAD$^+$, the interpretation of the time-dependent TRNOE data is relatively straightforward.

Thus, considering the adenosine moiety of NAD$^+$, the initial slopes of 1·72 s^{-1}, 1·47 s^{-1} and 0·33 s^{-1} for the decrease in intensity of the averaged H_A8 resonance following irradiation of the averaged H_A2', $H_A3'/H_N2'/H_N4'$ and H_A1'/H_N1' resonances, respectively, can be accounted for by direct cross-relaxation, in order of decreasing magnitude, between the H_A8 proton and the H_A2', H_A3' and H_A1' protons, characteristic of an *anti* conformation about the glycosidic bond. The presence of an initial lag phase in the intensity of the averaged H_A8 resonance following irradiation of the averaged $H_A5'/H_A5''/H_N5'$ resonance indicates that both the H_A5' and H_A5'' protons are separated by $\gtrsim$4 Å from the H_A8 proton so that the conformation about the C_A4'–C_A5' bond must be g^+. Qualitatively the same results were obtained with 5′-AMP.

Similarly, in the case of the nicotinamide riboside moiety of NAD$^+$, the initial slopes of 2·94 s^{-1}, 1·67 s^{-1}, 0·96 s^{-1} and 0·36 s^{-1} for the decrease in intensity of the averaged H_N6 resonance following irradiation of the averaged $H_A3'/H_N2'/H_N4'$, $H_A4'/H_N3'/H_N5'$, $H_A5'/H_A5''/H_N5''$ and H_A1'/H_N1' resonances, respectively, and the initial slope of 2·17 s^{-1} for the decrease in intensity of the averaged H_N2 resonance following irradiation of the averaged H_A1'/H_N1' resonance, can be accounted for by direct cross-relaxation, in order of decreasing magnitude, between the H_N6 proton and the H_N2', H_N3', H_N5'' and H_N1' protons, and by

TABLE 1

Initial slopes of the TRNOEs observed in the NAD$^+$–SDHase system with a ratio of free to bound NAD$^+$ of 12

Irradiated averaged ligand resonance	Initial slope of TRNOE on observed averaged ligand resonances (s^{-1})					
	H_A8	H_A2/H_N5	H_N6	H_N4	H_N2	H_A1'/H_N1'
H_A1'/H_N1'	0·33	lag	0·36	lag	2·17	—
H_A2'	1·72	lag	lag	lag	lag	1·67
$H_A3'/H_N2'/H_N5'$	1·47	1·64	2·94	lag	lag	2·18
$H_A4'/H_N3'/H_N5'$	0·72	1·66	1·67	lag	lag	2·18
$H_A5'/H_A5''/H_N5''$	lag	lag	0·96	lag	lag	1·86
H_A2/H_N5	lag	—	1·85	1·47	lag	lag

The experimental conditions are the same as those given in the Fig. 2 legend.

direct cross-relaxation between the H_N2 proton and the H_N1' proton. These observations are characteristic of an *anti* conformation about the nicotinamide glycosidic bond, and a t conformation about the C_N4'–C_N5' bond.

The initial slopes of $1{\cdot}67\ s^{-1}$ and $2{\cdot}18\ s^{-1}$ in the decrease in intensity of the averaged H_A1'/H_N1' resonance following irradiation of the averaged H_A2' and $H_A3'/H_N2'/H_N4'$ resonances are simply accounted for by direct cross-relaxation between the H_A1' and H_A2' protons and between the H_N1' and H_N2' protons.

Bearing the above observations in mind, we are left to account for three further observations.

(1) The initial slopes of $1{\cdot}66\ s^{-1}$ and $1{\cdot}64\ s^{-1}$ for the decrease in intensity of the averaged H_A2/H_N5 resonance following irradiation of the averaged $H_A4'/H_N3'/H_N5'$ and $H_A3'/H_N2'/H_N4'$ resonances, respectively.

(2) The initial slopes of $2{\cdot}18\ s^{-1}$ and $1{\cdot}86\ s^{-1}$ for the decrease in intensity of the averaged H_A1'/H_N1' resonance following irradiation of the averaged $H_A4'/H_N3'/H_N5'$ and $H_A5'/H_A5''/H_N5''$ resonances, respectively.

(3) The larger value for the initial slope of the TRNOE on the averaged H_N6 resonance ($1{\cdot}85\ s^{-1}$) than for that on the averaged H_N4 resonance ($1{\cdot}47\ s^{-1}$) following irradiation of the averaged H_A2/H_N5 resonance, given that the two distances $r_{H_N6\text{-}H_N5}$ and $r_{H_N4\text{-}H_N5}$ are both equal (2·46 Å) and no difference in correlation time for the H_N6–H_N5 and H_N4–H_N5 distance vectors would be expected.

Given the glycosidic bond and C4′–C5′ bond conformations of the adenosine and nicotinamide riboside moieties of bound NAD^+ deduced above from the time-

FIG. 4. Conformation of NAD^+ bound to SDHase deduced from model building on the basis of the interproton distances between pairs of bound ligand protons given in Table 2 derived from the time-dependent TRNOE measurements. (See Table 3 and the text for further details.)

TABLE 2

Cross-relaxation rates between pairs of bound ligand protons in the NAD$^+$–SDHase and 5′-AMP–SDHase complexes determined from time-dependent TRNOE measurements together with the interproton distance ratios and distances derived from them

	NAD$^+$			5′-AMP		
Proton pair	$\sigma_{i_a j_a}$ (s^{-1})[a]	Distance ratios[b]	$r_{i_a j_a}$ (Å)[c]	$\sigma_{i_a j_a}$ (s^{-1})[a]	Distance ratios[b]	$r_{i_a j_a}$ (Å)[c]
Intra-nicotinamide riboside moiety[d]		$r_{H_N2\ H_N1'}/r_{i_a j_a}$				
H_N1'–H_N6	4·3	0·74	3·11			
H_N1'–H_N2	26·1	1·00	2·30			
H_N1'–H_N2'	26·2	1·00	2·30			
H_N2'–H_N6	35·0	1·05	2·19			
H_N3'–H_N6	20·0	0·96	2·41			
H_N5''–H_N6	11·5	0·87	2·64			
H_N5–H_N4	17·6	0·94	2·46			
H_N5–H_N6[f]	17·6	0·94	2·46			
Intra-adenosine moiety[e]		$r_{H_A8\ H_A2}/r_{i_a j_a}$			$r_{H_A8\ H_A2}/r_{i_a j_a}$	
H_A1'–H_A8	4·0	0·76	3·15	[e]	—	—
H_A2'–H_A8	20·7	1·00	2·39	42·0	1·00	2·13
H_A3'–H_A8	17·6	0·97	2·46	11·1	0·80	2·66
H_A1'–H_A2'	20·0	0·99	2·41	18·2	0·87	2·45
Inter nicotinamide riboside–adenosine moieties		$r_{H_N2'\ H_A2}/r_{i_a j_a}$				
H_N2'–H_A2	19·7	1·00	2·41			
H_N3'–H_A2	19·9	1·01	2·41			
H_N6–H_A2[f]	4·4	0·78	3·10			
H_N5'–H_A1'	26·2	1·05	2·30			
H_N5''–H_A1'	22·3	1·02	2·36			

[a] The cross-relaxation rates ($\sigma_{i_a j_a}$) are calculated from the initial slopes of the TRNOEs using equation (1). The relative errors, $\Delta\sigma_{i_a j_a}/\sigma_{i_a j_a}$, in the values of the cross-relaxation rates are $\lesssim \pm 0{\cdot}10$.

[b] The interproton distance ratios are calculated using equation (4). The errors in the values of the distance ratios are $\lesssim \pm 0{\cdot}03$.

[c] The interproton distances are calculated using equation (2) taking the cross-relaxation rate and distance between the H_N5 and H_N4 protons as a reference, and assuming a single correlation time for all interproton distance vectors. The distance $r_{H_N5\text{-}H_N4}$, calculated on the basis of standard bond lengths and angles for the nicotinamide ring, has a value of 2·46 Å. Assuming an error of $\pm 0{\cdot}05$ Å in this estimate, the errors on the values of the other interproton distances are $\lesssim \pm 0{\cdot}15$ Å. The correlation time for the H_N5–H_N4 vector in bound NAD$^+$, calculated from the cross-relaxation rate and distance between the bound H_N5 and H_N4 protons is $\sim 7 \times 10^{-8}$ s. This value is within the range (6×10^{-8} to 10×10^{-8} s) predicted for the rotational diffusion time of a protein of $M_r \sim 150{,}000$ on the basis of the Stokes–Einstein equation.

[d] The TRNOEs between the following pairs of protons were characterized by a lag phase indicating a separation of $\gtrsim 4$ Å: H_N2' and H_N2, H_N3' and H_N2, H_N5' and H_N6, H_N5' and H_N2, H_N5'' and H_N2.

[e] The TRNOEs between the following pairs of protons were characterized by a lag phase indicating a separation of $\gtrsim 4$ Å: in the case of both NAD$^+$ and 5′-AMP: H_A1' and H_A2, H_A2' and H_A2, H_A3' and H_A2, and H_A5'/H_A5'' and H_A2; in addition, in the case of 5′-AMP: H_A1' and H_A8.

[f] As the H_N5 and H_A2 proton resonances are superimposed, the cross-relaxation rates between the H_N5 and H_N6 protons and between the H_A2 and H_N6 protons were calculated from the initial slope of the TRNOE by assuming that the cross-relaxation rate between the H_N5 and H_N6 protons is the same as that between the H_N5 and H_N4 protons. This is a reasonable assumption as the distances $r_{H_N6\ H_N5}$ and $r_{H_N5\ H_N4}$ are both the same (2·46 Å) and no difference in the correlation time of these 2 distance vectors would be expected.

dependent TRNOE measurements, model building indicates, that to account for these three observations a conformation is required in which the bound NAD^+ is folded, but not stacked, with the H_A2 proton of the adenine ring, in close proximity to the H_N2', H_N3' and H_N6 protons of the nicotinamide riboside moiety, and the H_A1' proton of the adenosine sugar ring in close proximity to the H_N5' and H_N5'' of the nicotinamide sugar ring (see Fig. 4).

An alternative explanation is that the three above observations might be due to direct cross-relaxation between bound ligand protons and protons of the protein: in the case of observations (1) and (2) this would involve the H_A2, H_N5, H_A1' and/or H_N1' protons and α and/or β protons of the protein with resonance positions identical to the averaged $H_A4'/H_N3'/H_N5'$, $H_A3'/H_N2'/H_N4'$ and $H_A5'/H_A5''/H_N5''$ resonances; in the case of observation (3) this would involve the H_N6 proton and a histidine C(2)H proton with a resonance position identical to the averaged H_A2/H_N5 resonance. However, such an explanation is regarded as unlikely on two counts: (i) in the case of 5'-AMP in the presence of SDHase no direct TRNOEs were observed either on the averaged H_A2 or H_A1' resonances following irradiation of the averaged H_A3', H_A4' or H_A5'/H_A5'' resonances; and (ii) in the case of SDHase alone no resolved histidine C(2)H resonances could be detected in the region of the averaged H_A2/H_N5 resonance which lies downfield

TABLE 3

Structural parameters describing the conformation of NAD^+ and 5'-AMP bound to SDHase determined by model building on the basis of the interproton distance ratios and distances given in Table 2

Conformational parameters	NAD^+	5'-AMP
Torsion angles[a]		
χ_A	−75° (*anti*)	−70° (*anti*)
δ_A	80° (C3'-*endo*)	100° (O1'-*endo*)
γ_A	g^+	g^+
θ_A	g^+	
ψ_A	g^-	
P_A	g^+ to t	
P_N	t	
ψ_N	g^+ to g^-	
θ_N	t	
γ_N	t	
δ_N	110° (C1'-*exo*)	
χ_N	−110° (*anti*)	
Distances		
C_A6–C_N2 (Å)	~8·5	
N_A1–N_N4 (Å)	~6·5	
Angle between the planes of the nicotinamide and adenine rings	~120°	

[a] The glycosidic bond torsion angles are defined as follows: $\chi_A = O_A1' - C_A1' \overset{\gamma_A}{=} N_A9 - C_A4$ for adenosine; $\chi_N = O_N1' - C_N1' \overset{\gamma_N}{=} N_N1 - C_N2$ for nicotinamide riboside. The other torsion angles are defined by: $O_A3' - C_A3' \overset{\delta_A}{=} C_A4' \overset{\gamma_A}{=} C_A5' \overset{\theta_A}{=} O_A5' \overset{P_A}{=} P_A \overset{\psi_A}{=} O_{pp} \overset{P_N}{=} P_N \overset{\theta_N}{=} O_N5' \overset{\gamma_N}{=} C_N5' \overset{\delta_N}{=} C_N4' \overset{\delta_N}{=} C_N3' - O_N3'$. The error in the determination of χ_A, χ_N, δ_A and δ_N is $\sim \pm 5°$.

from the main aromatic protein proton resonance envelope. It should be noted that this does not mean that there are no histidine C(2)H resonances in this region but simply that they are broadened beyond detection due to the large molecular weight of the protein; such broad resonances cannot be completely saturated so that even if direct cross-relaxation between the H_A2 and/or H_N5 proton and a histidine C(2)H proton was effective, the TRNOE would be expected to exhibit a lag phase.

To obtain a more detailed picture of the conformation of NAD^+ bound to SDHase we have calculated the cross-relaxation rates between pairs of bound ligand protons from the values of the initial slopes of the time-dependent TRNOEs using equation (1). Interproton distances were then calculated from these cross-relaxation rates using equation (2) and taking the cross-relaxation rate and distance between the H_N4 and H_N5 nicotinamide protons as a reference. These data together with those obtained for 5'-AMP bound to SDHase are given in Table 2. From the interproton distance data, the values for the glycosidic bond and C3'–C4' bond torsion angles, the range of the other torsion angles, the values for the distances between the C_A6 and C_N2 atoms and between the N_A1 and N_N4 atoms, and the angle between the planes of the nicotinamide and adenine rings can be determined by model building and are collected in Table 3.

4. Discussion

Hydrogen transfer is stereospecific in dehydrogenases requiring nicotinamide coenzymes, and involves either the 4-pro-R position (A-side) or 4-pro-S position (B-side) of the nicotinamide ring (for a review see Jeffrey, 1982). High-resolution crystal structures of NAD^+–enzyme complexes are available for three A-side specific dehydrogenases (lactate dehydrogenase, horse liver alcohol dehydrogenase and soluble malate dehydrogenase) as well as two forms of a B-side specific enzyme (glyceraldehyde-3-phosphate dehydrogenases from lobster and *Bacillus stearothermophilus* (Chandrasekhar *et al.*, 1973; Abdallah *et al.*, 1975; Webb *et al.*, 1973; Moras *et al.*, 1975; Biesecker *et al.*, 1977)).

The main X-ray investigations (for a review see Brändén & Eklund, 1980) together with recent work on horse liver alcohol dehydrogenase (Eklund *et al.*, 1981,1982*a*,*b*) have established the principal conformational features of the bound coenzyme. These are summarized in Table 4 and can be compared with the n.m.r. findings for yeast and horse alcohol dehydrogenases (Gronenborn & Clore, 1982*a*) and the present findings for SDHase. Six main points deserve comment.

(1) The n.m.r. and crystallographic findings show a considerable measure of agreement in the one case where both are available (horse alcohol dehydrogenase).

(2) For the two alcohol dehydrogenases (n.m.r. findings), the NAD^+ conformation is generally similar though not identical, and these are clearly related proteins, which do not have a high sequence homology (Jörnvall, 1977*b*).

(3) The NAD^+ conformations in lactate dehydrogenase, soluble malate dehydrogenase and glyceraldehyde-3-phosphate dehydrogenases are similar to those in the two alcohol dehydrogenases though there is no proven genetic relationship between them and the alcohol dehydrogenases.

TABLE 4

Comparison of the conformation of NAD$^+$ bound to SDHase with those of NAD$^+$ bound to other dehydrogenases

	SDHase (n.m.r. findings)[a]	Yeast and horse alcohol dehydrogenase (n.m.r. findings)[b]	Crystallized dehydrogenases[c]
Adenosine moiety			
Glycosidic bond	*anti*	*anti*	*anti*
Sugar pucker	C3'-*endo*	C3'-*endo*	C3'-*endo*
C_A4'–C_A5' bond	g^+	g^- or t	g^- or t
Nicotinamide riboside moiety			
Glycosidic bond	*anti*	*anti*	*anti* in A-type *syn* in B-type
Sugar pucker	C1'-*exo*	C3'-*endo*	C2'-*endo* to C3'-*endo*
C_N4'–C_N5' bond	t	g^+	g^+, g^- or t
Overall conformation of NAD$^+$[d]			
Overall conformation	Folded but not stacked	[e]	Extended
$r_{C_A8\,C_N2}$ (Å)	~8·5	—	14–16
$r_{N^1N_N4}$ (Å)	~6·5	—	16–18
Angle between the planes of the nicotinamide and adenine rings	~120°	—	~90°

[a] From this paper.

[b] From Gronenborn & Clore (1982*a*).

[c] Crystallographic data: lactate dehydrogenase (Chandrasekhar *et al.*, 1973; White *et al.*, 1976; Grau *et al.*, 1981); soluble malate dehydrogenase (Webb *et al.*, 1973); horse liver alcohol dehydrogenase (Abdallah *et al.*, 1975; Eklund *et al.*, 1981,1982*a*,*b*); glyceraldehyde-3-phosphate dehydrogenase from lobster (Moras *et al.*, 1975) and *B. stearothermophilus* (Biesecker *et al.*, 1977).

[d] A comparison of the backbone torsion angles (with the exception of that about the C3'–C4' bond which defines the sugar pucker and that about the C4'–C5' bond) is difficult as these are quite varied.

[e] The n.m.r. data are consistent with an extended conformation as no TRNOEs between the nicotinamide riboside and adenosine moieties, indicative of a folded conformation, could be detected (Gronenborn & Clore, 1982*a*).

(4) The principal conformational difference in the case of glyceraldehyde-3-phosphate dehydrogenase (*syn* instead of *anti* about the nicotinamide glycosidic bond) makes the general conformational similarity compatible with the different stereospecificity (B-side instead of A-side).

(5) The overall NAD$^+$ conformation in the SDHase–NAD$^+$ binary complex is different despite clear primary structure homology to both yeast and horse alcohol dehydrogenase (Jörnvall *et al.*, 1983). However, the glycosidic bond and ribose conformations for both the adenosine and nicotinamide riboside moieties of NAD$^+$ in the SDHase–NAD$^+$ binary complex are similar to those of NAD$^+$ bound to the other A type dehydrogenases.

(6) Insofar as the other dehydrogenases all give a "consensus" conformation for dehydrogenase-bound NAD$^+$ (extended with the planes of the nicotinamide and adenine rings 12 to 14 Å apart and approximately perpendicular to each other),

the different conformation in SDHase (folded but not stacked with the planes of the nicotinamide and adenine rings 6 to 8 Å apart and at about 120° to each other) is properly designated "unusual". This unusual conformation can, however, be converted into the conventional extended conformation by a simple change in the conformation about the C_A5'–O_A5' (θ_A) bond from g^+ to t. It is also important to stress that the present n.m.r. data relate to the binary complex and not the active ternary complex, and thus do not exclude the possibility that NAD^+ adopts the conventional extended conformation in the active ternary complex of SDHase.

SDHase has a distinctive primary structure (Jeffery *et al.*, 1984) much of which resembles both yeast and horse alcohol dehydrogenases about as closely as these alcohol dehydrogenases resemble one another (Jörnvall *et al.*, 1983). The altered overall conformation of bound NAD^+ in the binary complex as well as the different specificity of SDHase affords excellent opportunities for defining structure–function relationships among these proteins. The structural differences here are more complex than the single point mutation of the p21 protein associated with bladder cancer, mentioned in the Introduction (Tabin *et al.*, 1982; Taparowsky *et al.*, 1982). Nevertheless, many residues that interact with the coenzyme in horse alcohol dehydrogenase are conserved in SDHase (Jörnvall *et al.*, 1984), and the explanation of the different conformation of the bound NAD^+ in the binary complex is not simple to understand and may lie in the structure of the interdomain region. Thus, the present study demonstrates the need for cautious interpretation, as well as the great potential of the TRNOE technique.

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