J. Mol. Biol. (1982) **157**, 155–160

Conformation of NAD^+ Bound to Yeast and Horse Liver Alcohol Dehydrogenase in Solution

The Use of the Proton–Proton Transferred Nuclear Overhauser Enhancement

The glycosidic bond torsion angles and the conformations of the two riboses of NAD^+ bound to yeast and horse liver alcohol dehydrogenase in solution have been determined by a novel method involving the measurement of proton–proton transferred nuclear Overhauser enhancements by ^{1}H-nuclear magnetic resonance. In both cases the conformation of the adenosine and nicotinamide ribose is 3′-*endo* of the N type. The conformations about both glycosidic bonds are anti with χ_A (0-4′–C-1′–N-9–C-4) ~ 270° and 240° for yeast and horse liver alcohol dehydrogenase, respectively, and χ_N (0-4′–C-1′–N-1–C-2) ~ 240° for both yeast and horse liver alcohol dehydrogenase.

X-ray crystallographic studies of coenzymes and coenzyme fragments bound to a number of dehydrogenases have shown that one of the common qualitative features is an anti-conformation about both the adenosine and nicotinamide glycosidic bonds (Webb *et al.*, 1973; Chandrasekhar *et al.*, 1973; Abdallah *et al.*, 1975; White *et al.*, 1976; Samama *et al.*, 1977; Grau *et al.*, 1981). However, there are quite substantial differences in the actual values of the glycosidic bond torsion angles ranging from $\chi_A = 235°$ to 280° and $\chi_N = 200°$ to 270° for the adenosine and nicotinamide glycosidic bonds respectively†. In the present study we have determined the glycosidic bond torsion angles and the conformations of the two riboses of NAD^+ bound to horse liver and yeast alcohol dehydrogenase in solution by a novel method involving the measurement of proton–proton transferred nuclear Overhauser enhancements by ^{1}H-nuclear magnetic resonance. In both cases no crystallographic data are available.

The proton–proton nuclear Overhauser enhancement is the most direct nuclear magnetic resonance method of conformational analysis in solution as it can be used to demonstrate the proximity in space of two protons and to determine the distance between them (for an extensive review, see Noggle & Schirmer, 1971). NOE‡ techniques have been applied extensively to the conformational analysis of small molecules and more recently of small proteins (see, for example, Schirmer *et al.*, 1972; Jones *et al.*, 1978; Krishna *et al.*, 1978; Hart, 1978; Albrand *et al.*, 1979;

† The convention used for defining χ is the standard one adopted by Davies (1978): χ_A = 0-4′–C-1′–N-9–C-4 and χ_N = 0-4′–C-1′–N-1–C-2. χ is related to the angle χ' defined by Arnott & Hukins (1969) and adopted by most crystallographers by the relation $\chi = 360 - \chi'$.

‡ Abbreviations used: NOE, nuclear Overhauser enhancements; TRNOE, transferred nuclear Overhauser enhancements.

0022–2836/82/130155–06 $03.00/0 © 1982 Academic Press Inc. (London) Ltd.

Poulsen *et al.*, 1980; Kuo & Gibbon, 1980; Wagner *et al.*, 1981; Kumar *et al.*, 1981). In a non-exchanging system, the magnitude of the proton–proton NOE obtained on irradiating the resonance of proton j for a duration $t \rightarrow \infty$ and observing the intensity of the resonance of proton i is given by $(I_{i0} - I_{i\infty})/I_{i0} = -\sigma_{ij}/\rho_i$ where I_{i0} and $I_{i\infty}$ are the intensities of the resonance of proton i before and after irradiation of the resonance of proton j, σ_{ij} is the cross relaxation rate relating to the transfer of magnetisation between the two protons i and j, and ρ_i is the total spin-lattice relaxation rate of proton i. Quantitative conformational analysis can be obtained because σ_{ij} is inversely proportional to r_{ij}^6, the sixth power of the distance between the two protons. Consequently, the ratio of two distances r_{ij}/r_{ik} can be obtained from the ratio of the NOE values from protons k and j to proton i, N_{ik}/N_{ij}, from the relation $r_{ij}/r_{ik} = (\sigma_{ik}/\sigma_{ij})^{1/6} = (N_{ik}/N_{ij})^{1/6}$, providing tumbling of the molecule is isotropic and the two distance vectors are described by the same correlation time. For small ligand molecules with very short correlation times ($\tau_c < 10^{-10}$ s) for which $\omega\tau_c \ll 1$, positive proton–proton NOE values will be observed with a maximum value of +0·5; for protein–ligand complexes with long correlation times ($\tau_c > 10^{-8}$ s) for which $\omega\tau_c \gg 1$, negative proton–proton NOE values will be observed with a maximum value of −1·0.

The proton–proton TRNOE involves the extension of NOE measurements to exchanging systems such as protein–ligand complexes which can be described by the simple scheme $E + L \rightleftharpoons EL$ where E and L are the free protein and ligand respectively, and EL is the protein–ligand complex. The principles and theory of the TRNOE have recently been discussed in detail (Clore & Gronenborn, 1982) and will only be summarised briefly here. The basis of the TRNOE involves the transfer of magnetic information concerning cross relaxation between two bound ligand protons from the bound state to the free state by chemical exchange between the free and bound ligand so that negative TRNOE values on the easily detectable free or averaged ligand resonances† may be seen following irradiation of other ligand resonances (free, bound or averaged), thus conveying information on the proximity in space of bound ligand protons. The TRNOE technique is ideally suited to study conformations of small ligands bound to very large proteins as (1) it does not require the observation of individual protein or bound ligand resonances, and (2) its normalised magnitude increases as the molecular weight of the protein increases, reaching its maximum value when $(1-a)\rho_B \gg a\rho_F$ and $k > 1000\,\rho_F$, where ρ_F and ρ_B are the total spin-lattice relaxation rates (i.e. including cross relaxation terms) of the proton of interest in the free and bound states, respectively, a is the mole fraction of free ligand, and k the chemical exchange rate between free and bound states.

NAD^+ binds weakly ($K_a \lesssim 2 \times 10^3$ M^{-1}; Subramanian & Ross, 1977) to both yeast and horse liver alcohol dehydrogenase and exchange between free and bound NAD^+ is fast on the chemical shift scale so that only a single set of averaged ligand resonances is seen. Thus, the TRNOE experiments involve irradiation of one

† When chemical exchange between the free and bound ligand is slow on the chemical shift scale, two distinct sets of ligand resonances will be observed, one for the free ligand and the other for the bound ligand. When, however, chemical exchange is fast on the chemical shift scale, only a single set of averaged ligand resonances will be observed.

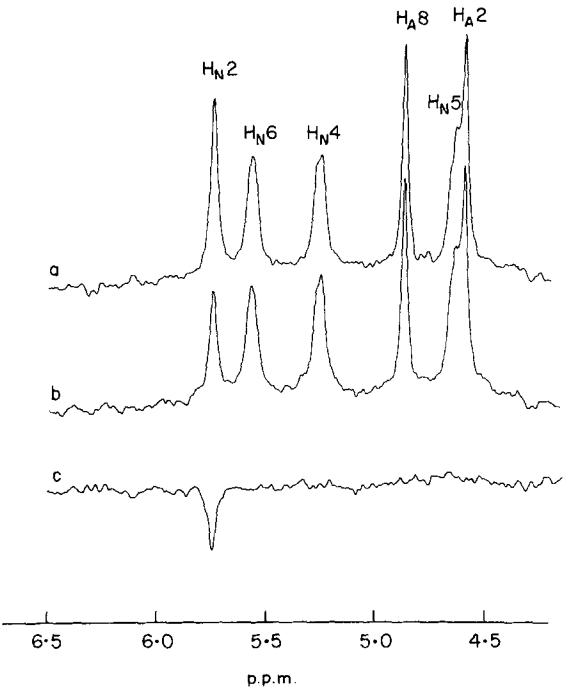

FIG. 1. The aromatic region of the 270 MHz ^{1}H-nuclear magnetic resonance spectrum of 3·33 mM-NAD^+ in the presence of 0·076 mM-yeast alcohol dehydrogenase (corresponding to 0·30 mM in NAD^+ binding sites): trace a, control irradiation at 3·25 p.p.m. (parts per million); trace b, irradiation of the H_N1' sugar resonance at 2·26 p.p.m.; trace c, spectrum b minus spectrum a. The free and bound states of NAD^+ are in fast exchange on the chemical shift scale so that there is only a single set of averaged ligand resonances whose positions, under these experimental conditions, are approximately at the positions of the corresponding resonances in free NAD^+.

Conditions of nuclear magnetic resonance spectroscopy: ^{1}H-nuclear magentic resonance spectra were obtained at 270 MHz using a Bruker WH 270 spectrometer in the Fourier transform mode with the pulse sequence $(t_1 - t_2 - \pi/2 - AT)_n$ where the selective irradiation at a chosen frequency was applied during the time interval t_1 (0·5 s), t_2 is a short delay (2 ms) to allow for electronic recovery after removal of the selective irradiation, and AT is the acquisition time (0·487 s). 500 transients, obtained by quadrature detection with 4096 data points for a spectral width of 4·2 KHz, were averaged for each spectrum. Before Fourier transformation, the free induction decay was multiplied by an exponential equivalent to a line broadening of 2 Hz. Chemical shifts are expressed relative to internal (1 mM) dioxan (3·71 p.p.m. downfield from 2,2 dimethyl silapentane-5-sulphonate). The sample temperature was 20°C. Experimental conditions: yeast and horse liver alcohol dehydrogenases were purchased from Sigma Chemicals Co. Ltd. After extensive dialysis against 20 mM-potassium phosphate (pH* 7·0) (meter reading uncorrected for the isotope effect on the glass electrode) in D_2O, the solutions were clarified by centrifugation and used without further purification. NAD^+ was also obtained from Sigma Chemicals Co. Ltd. lyophilised from D_2O, and used without further purification. All chemicals used were of the highest purity commercially available.

averaged ligand resonance and monitoring the intensities of the others. Figure 1 illustrates such an experiment for the NAD^+-yeast alcohol dehydrogenase system. At an approximately tenfold molar excess of free over bound NAD^+, a large negative TRNOE of -35% is seen on the resonance of the 2 proton of the nicotinamide ring (H_N2) following irradiation of the 1′ proton of the nicotinamide sugar ring (H_N1'). This effect is highly specific, and therefore excludes a non-specific

TABLE 1

TRNOE measurements on the adenine moiety of NAD$^+$ in the presence of horse liver and yeast alcohol dehydrogenase

Irradiated resonance	Observed resonance	% TRNOE Horse liver ADH (M_r = 80,000)	% TRNOE Yeast ADH (M_r = 150,000)
H_A1'	H_A8	0	0
H_A2'	H_A8	−20	−42
H_A3'	H_A8	−9	−21
H_A5'/H_A5''[b]	H_A8	−7	−9
Conformation about the adenosine glycosidic bond		Anti	Anti
$'H_A8–H_A2''/''H_A–H_A3'$		0·86	0·89
$'H_A8–H_A2''/''H_A8–H_A5'/H_A5''$†		0·84	0·77
χ_A(O-4′–C-1′–N-9–C-4)		~ 240°	~ 270°
Adenosine ribose		3′-*endo*	3′-*endo*

Normalised magnitude of the TRNOE values measured on the resonance of the H_A8 proton of the adenine ring following irradiation of the resonance of the adenosine sugar protons in the presence of horse liver and yeast alcohol dehydrogenase (ADH), together with the distance ratios, values of the glycosidic bond torsion angle and conformation of the adenosine ribose of bound NAD$^+$. TRNOE measurements were carried out systematically at 20 Hz intervals throughout the entire sugar proton region of the spectrum, using the pulse sequence given in the legend to Fig. 1. The ratio of the concentration of free to bound NAD$^+$ was approx. 10.

No TRNOE values were detected on the resonance of the H_A2 proton of the adenine ring following irradiation of any of the adenosine sugar proton resonances.

† The resonance of the H_A5' and H_A5'' sugar protons are superimposed so that we cannot distinguish whether the TRNOE to the H_A8 proton arises from the H_A5' or H_A5'' proton.

TABLE 2

TRNOE measurements on the nicotinamide moiety of NAD$^+$ in the presence of horse liver and yeast alcohol dehydrogenase

Irradiated resonance	Observed resonance	% TRNOE Horse liver ADH (M_r = 80.000)	% TRNOE Yeast ADH (M_r = 150,000)
H_N1'	H_N2	−13	−35
H_N2'	H_N6	0	0
H_N3'	H_N6	−15	−39
H_N5'/H_N5''	H_N6	0	0
Conformation about the nicotinamide glycosidic bond		Anti	Anti
χ_N(O-4′–C-1′–N-1–C-2)		~ 240°	~ 240°
Nicotinamide ribose		3′-*endo*	3′-*endo*

Normalised magnitude of the TRNOE values measured on the resonance of the H_N2 and H_N6 protons of the nicotinamide ring following irradiation of the resonances of the nicotinamide sugar protons in the presence of horse liver and yeast alcohol dehydrogenase (ADH), together with the values of the glycosidic bond torsion angle and the configuration of the nicotinamide ribose for bound NAD$^+$ deduced from them. TRNOE measurements were carried out systematically at 20 Hz intervals throughout the entire sugar proton region of the spectrum, using the pulse sequence given in the legend to Fig. 1. The ratio of the concentration of free to bound NAD$^+$ was approx. 10.

spin diffusion mechanism. The results of TRNOE experiments carried out by systematic irradiation at 20 Hz intervals throughout the entire sugar proton region of the spectrum, are summarised in Tables 1 and 2 for the adenosine and nicotinamide moieties of NAD^+, respectively. For the complexes of NAD^+ with both yeast and horse liver alcohol dehydrogenase, it is clear that the conformations of the glycosidic bonds of the adenosine and nicotinamide rings are entirely anti, as the only TRNOE values observed in the case of the adenosine moiety are from the H_A2', H_A3' and H_A5'/H_A5'' protons of the adenosine sugar to the H_A8 proton of the adenine ring, and in the case of the nicotinamide moiety from the H_N1' and H_N3' sugar protons to the H_N2 and H_N6 protons of the nicotinamide ring, respectively.

Quantitative determination of distance ratios may be obtained by taking the sixth root of the inverse ratio of the TRNOE values observed from two protons to a third proton providing the condition $|a\sigma_F| \ll |(1-a)\sigma_B|$ is satisfied, where σ_F and σ_B are the cross relaxation rates between the relevant pairs of protons in the free and bound states, respectively, and a is the mole fraction of free ligand, and the assumption of a single correlation time for the two distance vectors is made (Clore & Gronenborn, 1982). Under our experimental conditions no NOE values were observed for free NAD^+, either alone or in the presence of denatured yeast or horse liver alcohol dehydrogenase in 3 M-guanidinium chloride, so that the above condition is satisfied. From the distance ratios the glycosidic bond torsion angle and the ribose conformation can be determined by model-building. For both yeast and horse liver alcohol dehydrogenase, the calculated distances ratios are only consistent with a 3′-*endo* conformation of the N type for the adenosine ribose, and the values obtained for χ_A are 270° and 240°, respectively (see Table 1). Moreover, from the observation of a TRNOE from the H_A5'/H_A5'' sugar proton(s) to the H_A8 proton, we deduce that the conformation about the C-4′–C-5′ bond of the ribose is either *gauche–trans* or *trans–gauche* (we cannot distinguish between these two possibilities as the resonances of the H_A5'/H_A5'' protons are superimposed). These findings are consistent with all the available crystallographic data on coenzymes and coenzyme fragments bound to dehydrogenases.

In the case of the nicotinamide moiety, distance ratios could not be calculated as only a single TRNOE was observed to both the H_N2 and H_N6 protons of the nicotinamide ring. However, model-building shows that for a TRNOE to be observed from the H_N3' sugar proton (but not the H_N2' and H_N5'/H_N5'' protons) to the H_N6 proton, the conformation of the ribose must be 3′-*endo* of the N type, and given the observation of a TRNOE from the H_N1' sugar proton to the H_N2 proton, the value of χ_N has to be approximately 240° (see Table 2). It is interesting to note that the 3′-*endo* conformation of the N type for the nicotinamide ribose of NAD^+ bound to horse liver and yeast alcohol dehydrogenase deduced from these experiments is the same as that obtained by X-ray crystallography for NAD^+ bound to lactate dehydrogenase (Chandrasekhar *et al.*, 1973; White *et al.*, 1976), but contrasts to the 2′-*endo* conformation of the S type for NAD^+ bound to malate dehydrogenase (Webb *et al.*, 1973).

In our view the power of TRNOE measurements demonstrated here lies in its ability to determine rapidly and with ease the conformations of mono- and dinucleotides bound to large proteins in solution. However, this new technique does

not in any way replace X-ray crystallographic measurements which are able to determine the entire three-dimensional structure of such ligand–protein complexes. Nevertheless, the TRNOE measured by ^{1}H-nuclear magnetic resonance provides a powerful tool supplementing crystallographic studies, particularly in cases where crystal data are not available (as in the two cases considered here), or in comparative studies of ligand–protein complexes with an array of modified ligands.

We thank Sir Arnold Burgen for continual encouragement and support.

Division of Molecular Pharmacology
National Institute for Medical Research
Mill Hill, London NW7 1AA, England

A. M. GRONENBORN
G. M. CLORE

Received 4 January 1982

REFERENCES

Abdallah, M. A., Biellmann, J. F., Nordström, B. & Bränden, C.-I. (1975). *Eur. J. Biochem.* **50**, 475–481.

Albrand, J. P., Birdsall, B., Feeney, J., Roberts, G. C. K. & Burgen, A. S. V. (1979). *Int. J. Biol. Macromol.* **1**, 37–41.

Arnott, S. & Hukins, D. W. L. (1969). *Nature (London)*, **224**, 886–888.

Chandrasekhar, K., McPherson, A., Adams, M. J. & Rossmann, M. G. (1973). *J. Mol. Biol.* **76**, 503–512.

Clore, G. M. & Gronenborn, A. M. (1982). *J. Magn. Reson.* In the press.

Davies, D. B. (1978). *Prog. Nucl. Magn. Reson. Spectrosc.* **12**, 135–225.

Grau, U. M., Trommer, W. E. & Rossmann, M. G. (1981). *J. Mol. Biol.* **151**, 289–307.

Hart, P. A. (1978). *Biophys. J.* **24**, 833–861.

Jones, C. R., Sikakana, C. T., Hehir, S., Kuo, M.-C. & Gibbon, W. A. (1978). *Biophys. J.* **24**, 815–832.

Krishna, N. R., Agresti, D. G., Glickson, J. D. & Walter, R. (1978). *Biophys. J.* **24**, 791–814.

Kumar, A., Wagner, G., Ernst, R. E. & Wüthrich, K. (1981). *J. Amer. Chem. Soc.* **103**, 3654–3658.

Kuo, M.-C. & Gibbon, W. A. (1980). *Biophys. J.* **32**, 807–836.

Noggle, J. H. & Schirmer, R. E. (1971). *The Nuclear Overhauser Effect: Chemical Applications*, Academic Press, New York.

Poulsen, F. M., Hosch, J. C. & Dobson, C. M. (1980). *Biochemistry*, **19**, 2597–2607.

Samama, J.-P., Zeppezauer, E., Biellmann, J.-F. & Bränden, C.-I. (1977). *Eur. J. Biochem.* **81**, 403–409.

Schirmer, R. E., Davis, J. P., Noggle, J. H. & Hart, P. A. (1972). *J. Amer. Chem. Soc.* **94**, 2561–2572.

Subramanian, S. & Ross, P. D. (1977). *Biochem. Biophys. Res. Commun.* **78**, 461–466.

Wagner, G., Kumar, A. & Wüthrich, K. (1981). *Eur. J. Biochem.* **114**, 375–384.

Webb, L. E., Hill, E. J. & Banaszak, L. J. (1973). *Biochemistry*, **12**, 5101–5109.

White, J. L., Hackert, M. L., Buehner, M., Adams, M. J., Ford, G. C., Leutz, P. J., Smiley, I. E., Stiendel, S. J. & Rossmann, M. G. (1976). *J. Mol. Biol.* **102**, 759–779.

Edited by A. Klug