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BINDING OF NAD+ TO RABBIT-MUSCLE GLYCERALDEHYDEPHOSPHATE DEHYDROGENASE AS STUDIED BY OPTICAL ROTATORY DISPERSION AND CIRCULAR DICHROISM

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SUMMARY

1. The circular-dichroism spectra of both charcoal-treated and NAD+-containing glyceraldehydephosphate dehydrogenase from rabbit muscle were determined from 200 mμ to 500 mμ.

2. The difference in the optical rotatory dispersion of the holoenzyme and the apoenzyme was calculated from the circular-dichroism spectra by the Kronig–Kramers transformation.

3. The change in the Moffitt–Yang parameter, b_0, and the change in molecular ellipticity at 350 mμ of the enzyme were followed as a function of added NAD+. The curves obtained gave a linear increase up to 2 molecules of NAD+ per molecule of enzyme, and an intersection point at 3.0 molecules NAD+ per molecule enzyme.

4. By following the change in molecular ellipticity at 330 mμ on addition of 3-acetylpyridine–adenine dinucleotide, a similar result was obtained as with NAD+.

5. Circular dichroism curves with a maximum at 330 mμ were found both with the enzyme–NADH complex and the enzyme–NAD+ complex treated with iodoacetate.

6. Since binding of the enzyme with NAD+ has no effect on the optical rotatory dispersion or the circular dichroism below 250 mμ, it is concluded that the apo- and holoenzyme do not differ in overall protein conformation. The changes found above 250 mμ are at least partly due to extrinsic bands of the NAD+.

7. The broad band between 300 and 450 mμ in the circular-dichroism spectrum of holoenzyme is probably composed of at least two components: (i) the charge-transfer complex between NAD+ and the reactive –SH group of the protein; (ii) an interaction between the adenine moiety and the enzyme.

Abbreviation: APAD+, 3-acetylpyridine–adenine dinucleotide, oxidized form.

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INTRODUCTION

Rabbit-muscle glyceraldehydephosphate dehydrogenase (glyceraldehyde 3-phosphate:NAD^+ oxidoreductase (phosphorylating), EC 1.2.1.12) is a tetramer[1] and can combine with 4 molecules of NAD^+ (refs. 2, 3). KOSHLAND *et al.*[2] and DE VIJLDER AND SLATER[3] have shown that two molecules are bound very firmly, the third less firmly, and the fourth weakly. DE VIJLDER AND SLATER[3] showed that the three molecules more firmly bound to the enzyme cause the appearance of the absorption band at 360 mμ first observed by RACKER AND KRIMSKY[4], with each molecule contributing equally to the spectrum, whereas the fourth molecule makes little if any additional contribution to the spectrum. However, all four molecules are reduced by glyceraldehyde[3,5].

LISTOWSKY *et al.*[6] and BOLOTINA *et al.*[7] observed changes in the optical rotatory dispersion parameters of the Moffitt–Yang equation and the modified two-term Drude equation on addition of NAD^+ to the apoenzyme. It is not completely clear what the information obtained from these studies means. BOLOTINA *et al.*[7] interpreted these changes as indicating an increase of α-helix, but LISTOWSKY *et al.*[6] did not find any effect on optical activity between 200 and 250 mμ, the region of the Cotton effects characteristic for protein backbone conformation. Since they found an inflection point at 280 mμ in the optical rotatory dispersion difference spectrum (holoenzyme *minus* apoenzyme), they suggested that alterations in the induced optical activity of the aromatic amino acids brought about by changes in their spatial orientation were involved, although they could not exclude the possibility of an extrinsic Cotton effect of the bound NAD^+.

In agreement with the absorbance measurements[3], LISTOWSKY *et al.*[6] found that only 3 molecules of bound NAD^+ contribute to the changes in optical rotatory dispersion. However, in contrast with the absorbance data, the first molecule was found to cause a greater change than the other two. In this paper we examined this effect and studied the alterations in the optically active absorption bands by means of circular dichroism.

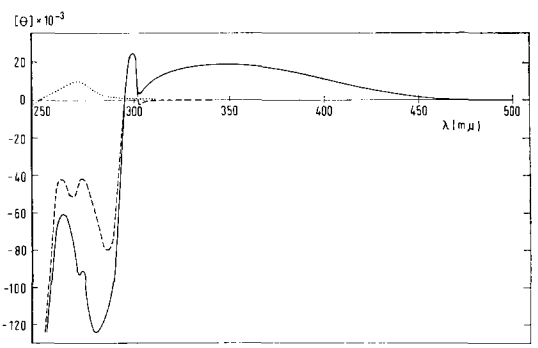


Fig. 1. Circular dichroism of glyceraldehydephosphate dehydrogenase, — — —, spectrum of the apoenzyme; ———, spectrum of the holoenzyme (7.5 moles added NAD^+ per mole of enzyme), corrected for free NAD^+ (3.5 moles free NAD^+ per mole of enzyme, as calculated from dissociation constants); · · · · ·, spectrum of NAD^+. 152 μM enzyme in 0.1 M Tris–HCl buffer (pH , 8.2) containing 2 mM EDTA.

RESULTS

The circular-dichroism spectra between 250 mμ and 500 mμ of charcoal-treated and NAD^+-containing enzyme are shown in Fig. 1. The apoenzyme gives a complicated spectrum in the absorption region of the aromatic amino acids. On addition of NAD^+ (7.5 molecules per molecule enzyme) a very broad band with a maximum at 350–360 mμ and a molecular ellipticity $[\Theta]$ of 18 000 (degrees·l·mole^{-1}·cm^{-1}) at 350 mμ, calculated per mole enzyme, was observed. The positive band at 299 mμ was not affected, but the negative bands were shifted slightly and intensified. At 220 mμ there was a broad negative band with $[\Theta] = 12 \cdot 10^6$, calculated per mole of enzyme. In this band no change was observed on addition of NAD^+. In agreement with LISTOWSKY *et al.*[6] the optical rotatory dispersion spectrum between 200 and 250 mμ was also unaffected by NAD^+. This spectrum showed a trough at 233 mμ with a reduced mean residue rotation $[m']_{233}$ of −3300.

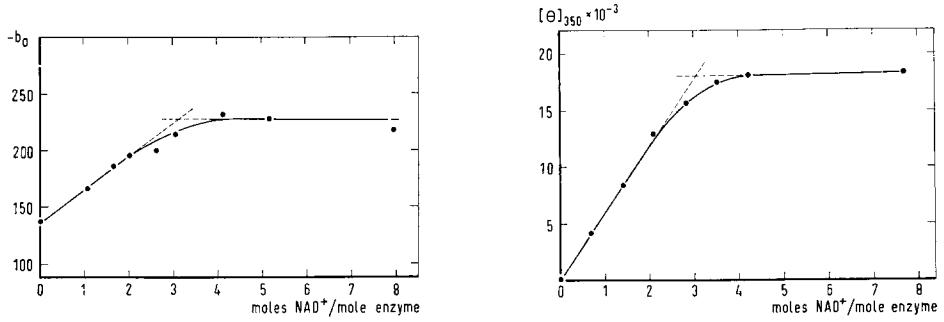


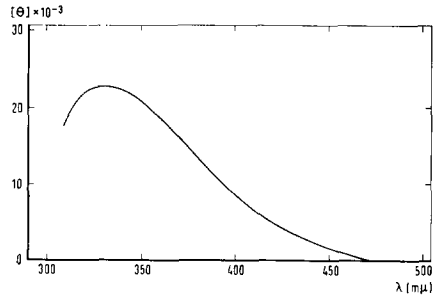
Fig. 2. Change of the Moffitt–Yang parameter, b_0, of glyceraldehydephosphate dehydrogenase (158 μM) upon adding NAD^+. The enzyme was dissolved in 0.1 M Tris–HCl buffer (pH 8.2), containing 2 mM EDTA.

Fig. 3. Changes in molecular ellipticity $[\Theta]$ at 350 mμ of glyceraldehydephosphate dehydrogenase (152 μM) on adding NAD^+. For conditions, see Fig. 2.

Fig. 2 shows the effect of increasing NAD^+ concentrations on the Moffitt–Yang parameter, b_0. The increase in $-b_0$ was linear with up to two molecules NAD^+ added per molecule enzyme. The intersection point of the titration curve was found at 3.0–3.1 molecules NAD^+ per molecule enzyme. This titration follows a similar course to that of the spectrophotometric titration[3], and differs from that reported by LISTOWSKY *et al.*[6]. A similar curve was also obtained when the titration was carried out by following the molecular ellipticity (Fig. 3).

The complex of the enzyme with 3-acetylpyridine–adenine dinucleotide ($APAD^+$) has a very broad absorption band[8] in which circular dichroism can be observed (Fig. 4). A maximal ellipticity of 23 000 per mole of enzyme was found at 330 mμ. Titration of the enzyme with $APAD^+$ by following the molecular ellipticity gave an intersection point at about 3 molecules $APAD^+$ per molecule enzyme (Fig. 5).

Addition of NADH to the enzyme–NAD^+ complex caused a shift of the maximum in the circular-dichroism curve from 360 mμ (Fig. 6, Curve b) to 330 mμ (Fig. 6,



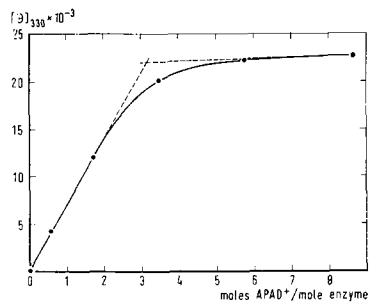


Fig. 4. Circular-dichroism spectrum of enzyme–APAD+ complex (1:8.6). Enzyme concentration, 158 μM. For conditions, see Fig. 2.

Fig. 5. Changes in molecular ellipticity [Θ] at 330 mμ of glyceraldehydephosphate dehydrogenase (158 μM) on adding APAD+. For conditions, see Fig. 2.

Curve a). The same position of the maximum was obtained with NADH alone, but [Θ] was only 16 000 compared with 24 000 in the presence of both NAD+ and NADH (Fig. 6, Curve c). NADH itself has only a very weak negative band at 340 mμ ([Θ] = −500).

Iodoacetate caused almost complete disappearance of the 360-mμ band, but a small residual dichroism at 320–330 mμ remained (Fig. 6, Curve d). Between 250 and 300 mμ, the circular-dichroism spectrum returned almost completely to that of the apoenzyme (not shown).

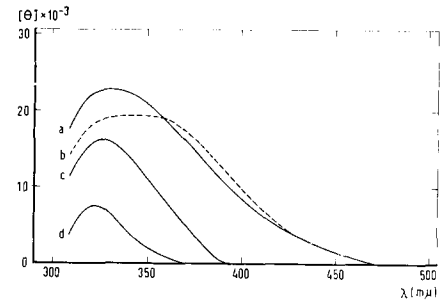


Fig. 6. Circular-dichroism spectrum of enzyme–NAD+–NADH (Curve a), enzyme–NAD+ (Curve b) and enzyme–NADH (Curve c) complexes. 7.5 moles NAD+ and/or NADH present per mole of enzyme (158 μM) dissolved in 0.1 M Tris–HCl buffer (pH 8.2) containing 2 mM EDTA. The enzyme used for Curve c contained 0.4 mole NAD+ per mole enzyme. Curve d, iodoacetate (20 mM) added to enzyme–NAD+ complex.

DISCUSSION

Our results agree with those of LISTOWSKY *et al.*[6] in showing no effect of binding of the enzyme with NAD+ on the optical rotatory dispersion in the far ultraviolet. Moreover, no effect on the circular dichroism was found. It seems then that the apo- and holoenzyme do not differ in overall protein conformation. However, it is still possible that conformation changes occur that cannot be observed by optical rotatory

dispersion or circular dichroism in this wavelength region. The changes in the Moffitt–Yang and Shechter and Blout parameters on NAD^+ binding are due to alterations in the optical activity of the aromatic amino acids (*cf.* ref. 6) or to an extrinsic effect of the NAD^+ or to both.

Since on addition of NAD^+ the circular dichroism spectrum between 250 mμ and 300 mμ is affected only in the region in which NAD^+ itself has a positive circular-dichroism band, it is quite possible that the changes in optical activity in this wavelength region are due to an extrinsic effect of NAD^+. The broad circular-dichroism band between 300 and 500 mμ in the holoenzyme is certainly caused by NAD^+ itself.

It is clear that all these changes in optical activity will influence the optical rotatory dispersion spectrum and hence will contribute to the alterations upon NAD^+ binding in the parameters derived from the Moffitt–Yang equation or the modified two-term Drude equation of Shechter and Blout. Fig. 7 shows the optical rotatory

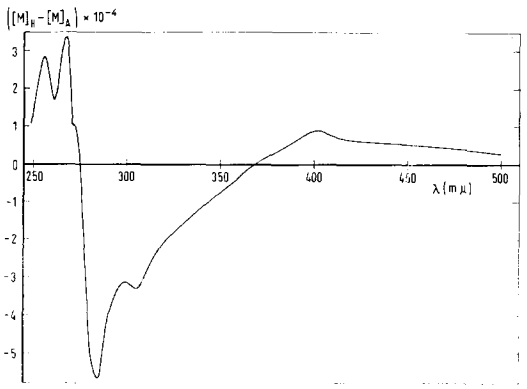


Fig. 7. Difference between the molecular rotation of the holoenzyme and the apoenzyme ($[M]_H - [M]_A$) as calculated from the measured circular dichroism by means of the Kronig–Kramers transformation.

dispersion difference spectrum (holo- *minus* apoenzyme) calculated from the circular-dichroism spectra by means of the Kronig–Kramers transformation. Since the difference is only a few percent of the rotation of the apoenzyme, the calculated difference spectrum is much more accurate than one that is determined directly, but no gross discrepancies between the two spectra were found. The values of $[M]_H - [M]_A$, both calculated and measured directly, are much lower than those reported by LISTOWSKY *et al.*[6].

The optical rotation between 300 and 500 mμ, the region in which the dispersion equations are normally used, is the sum of the rotations caused by the conformation of the protein backbone and the other optically active absorption bands. Since the latter are very small compared to the former, it is not surprising that no deviation from linearity was observed in the Moffitt–Yang or the Shechter and Blout plots.

According to KOSOWER[9], the absorption band at 360 mμ may be due to a charge-transfer complex between the thiol group of the reactive cysteine in the protein and the pyridine ring of the NAD^+. The broad band in this region in the circular-dichroism

spectrum must have the same origin. The band appears to be composed of more than one band, since the Kronig–Kramers transformation of the spectrum in this region revealed a multiple Cotton effect. NADH, even in the presence of NAD^+, causes the wavelength of maximum ellipticity to change from 360 mμ to 330 mμ. A maximum at the latter wavelength was also found in holoenzyme treated with iodoacetate. Therefore the 330-mμ band cannot be due to the charge-transfer complex, since this requires both NAD^+ and the active thiol group, and the latter reacts with iodoacetate[4]. It may be due to an interaction between the adenine moiety of NAD and the enzyme.

EXPERIMENTAL

Glyceraldehydephosphate dehydrogenase was isolated as described in a previous communication (*cf.* ref. 3) from rabbit muscle by the method of CORI *et al.*[10] slightly modified by HILVERS[11]. NAD^+ was removed by stirring with a 20% charcoal suspension (*cf.* ref. 12). The concentration of the enzyme was calculated from absorbance measurements at 280 mμ, using the extinction coefficients reported by FOX AND DANDLIKER[13]. A molecular weight of 145 000 was used for the enzyme[1].

Measurements of optical rotatory dispersion were made with a Bendix Polarmatic 62 and with a Jasco spectropolarimeter, Model ORD/UV/CD-5. The latter was also used for the circular-dichroism measurements. The curves of the holoenzyme were corrected for the small dilution caused by the addition of the coenzyme to the apoenzyme solution. Values of b_0 were obtained by analysis according to the Moffitt–Yang equation of the optical rotatory dispersion between 300 and 500 mμ, obtained with the Bendix instrument, equipped with an Osram 150-W xenon lamp. In this case a 10-mm cell was used, and the protein was dissolved to about 5 mg/ml in 0.1 M Tris buffer (pH 8.2) in the presence of 2 mM EDTA. No deviation from linearity was observed for the Moffitt plots between 300 and 500 mμ when a λ_0 of 212 mμ was used. Measurements of optical rotatory dispersion below 300 mμ and the circular-dichroism measurements were made with the Jasco instrument, provided with a 450-W xenon lamp. A pathlength of 0.1, 1 or 10 mm was used, and the enzyme concentration was 23 mg/ml or less. Because of the high absorbance of the Tris buffer in the far ultraviolet, for measurements below 250 mμ the enzyme was dissolved in a phosphate buffer with the same pH and concentration, containing 2 mM EDTA, or in a very dilute Tris buffer. On applying the Kronig–Kramers transformation we use an IBM 360-50 computer.

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