

Pressure, Temperature and pH Dependence of the Absorption Spectrum of Reduced Nicotinamide Adenine Dinucleotide

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Z. Naturforsch. **36 c**, 84–86 (1981); received October 9, 1980

Absorption, Dehydrogenases, High Pressure, NADH, Oxidoreductases, pH Dependence, Temperature Dependence

Enzymological studies at high hydrostatic pressure generally involve temperature, pH and pressure as variables, owing to the effect of adiabatic compression and the ionization volume of the buffer system. In the case of NAD dependent oxidoreductases this implies that the extinction coefficient of the coenzyme may be affected by p , T and pH, apart from the spectral change accompanying the redox reaction.

Measurements of the pressure dependence of the absorbance of NADH show a slight red shift and a 1% decrease (3% increase) of the absorbance at 339 nm (360 nm) at 2 kbar. The pH dependence at the given wavelengths amounts to $-(2.4 \pm 0.1)\%$ per pH unit (25 °C), while the intrinsic temperature effect (after correction for thermal expansion) is of the order of -0.2% per degree (20–30 °C).

Applying buffers with negligible ionization volume, 366 nm is the optimum wavelength for high pressure studies up to 2 kbar because here the pressure dependent spectral changes of the NADH absorption vanish.

Introduction

In recent years a number of studies on enzymes at high hydrostatic pressure have been performed in connection with questions concerning the structural stability of proteins on one hand, and mechanisms of adaptation to deep-sea conditions on the other.

Since NAD dependent dehydrogenases are among the best-known enzymes regarding their three-dimensional structure and the elementary processes involved in their catalytic mechanism, many high pressure experiments have been concerned with lactic dehydrogenase [1–5], glyceraldehyde-3-phosphate dehydrogenase [1], and glutamic dehydrogenase [6, 7].

The respective activity measurements under conditions of high pressure generally involve temperature and pH as additional variables, owing to the adiabatic compression and the reaction volume connected with the ionization of the buffer [8, 9].

For quantitative analyses of dehydrogenase activity under pressure the intrinsic effects of pressure, temperature and pH on the extinction coefficient of the coenzyme, NADH, has to be determined, independent of the well-known spectral changes during the redox reaction accompanying the catalytic reaction of the enzyme. The following measurements were performed to provide this information in

order to correct for the respective changes in high pressure studies on the structure-function relationship of NAD dependent dehydrogenases.

Materials and Methods

β -NADH was used as disodiumsalt, grade I (99% pure), as purchased from Boehringer (Mannheim). Buffer substances were of A grade purity (p.a. Merck, Darmstadt); bidistilled water was used.

Absorption spectra were measured in a Zeiss DMR 10 spectrophotometer, using a high pressure cuvette (pathlength 1.0 mm) according to Gaarz and Lüdemann [10], and quartz cuvettes (pathlength 10 mm) (Suprasil, Hellma, Müllheim/Baden). Data were corrected for the compressibility of water.

In order to avoid temperature effects in the high pressure experiments the pressure cell was thermostated with a water jacket at 25 °C. The influence of pressure on the pH was eliminated by choosing Tris buffer (0.01 M pH 8.0) as solvent; its ionization volume is about zero so that no pH shift is detectable in the given pressure range. To measure the pH dependence of the absorbance a stock solution of NADH and calibrated volumetric flasks were applied. Phosphate buffer with different pH ($I = 0.1$) was used to maintain constant pH in the range $5 \leq \text{pH} \leq 8$. The corresponding temperature dependent measurements at constant pH were performed

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0341-0382/81/0100-0084 \$ 01.00/0



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in a thermostated cuvette under nitrogen. The temperature was monitored within the cuvette using a miniature thermocouple (Philips CA 3 S 500).

Results and Discussion

As shown in Fig. 1 the effect of pressure on the absorption spectrum of NADH is small: after correcting for the compressibility of water, a decrease of the maximum absorbance at 339 nm of the order of 1% is observed at 2 kbar. Due to a slight red shift of the absorption band (insert, Fig. 1) the effect vanishes at about 350 nm; with decreasing absorbance at higher wavelengths it becomes more and more significant. At wavelengths commonly used for enzyme assays (340 and 366 nm) the pressure effect on the extinction coefficient amounts to -1% and $+3\%$, respectively.

The maximum absorbance of NADH is found to be shifted from 339 nm at normal pressure to 341 nm at 2 kbar; increasing temperature causes the opposite effect (*cf.* [11]).

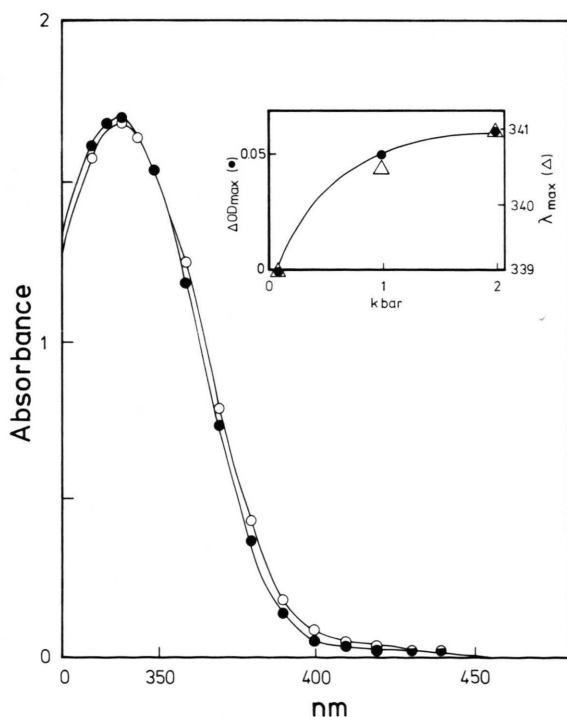


Fig. 1. Pressure dependence of the absorption spectrum of NADH in Tris buffer pH 8, 0.01 M; $c_{\text{NADH}} = 2.8 \text{ mM}$, 1 mm pathlength. (●) 10 bar, (○) 2 kbar. Insert: Absorbance change and red shift at varying pressure.

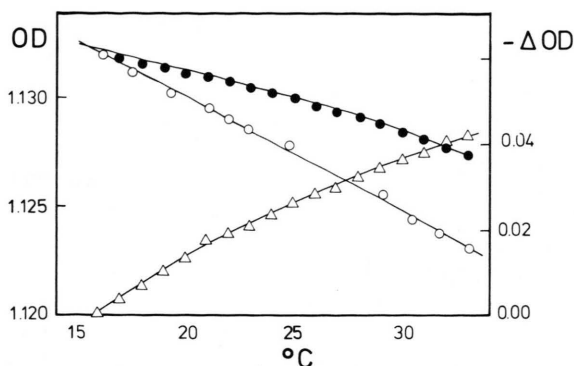


Fig. 2. Temperature dependence of the NADH absorption at 366 nm in water. ● calculated according to thermal expansion; ○ experimental data (OD) left ordinate; Δ true absorbance difference (ΔOD) right ordinate.

Pressure induced red shifts of this order of magnitude are commonly observed in the UV absorption of aromatic compounds in aqueous solution [12]. They can be explained in a semi-quantitative way by the influence of pressure upon the interaction between the permanent and the induced dipoles of the solvent and the solute on one hand and the different parts of the NADH molecules on the other [13].

The pH dependence of the NADH absorbance in the range between pH 5 and pH 8 is characterized by a linear decrease of $2.4 \pm 0.1\%$ per pH unit (25°C) [14]. This holds for constant wavelengths at 340 and 366 nm. Combining this result with the previously mentioned pressure effects, this means that both the pressure and pH induced spectral changes may compensate each other in a given buffer at certain wavelengths (*e.g.* phosphate buffer pH 7, $\sim 2 \text{ kbar}$, at 366 nm (*cf.* [8])). At 340 nm both effects run parallel so that significant corrections may occur.

For high pressure studies the wavelength of 366 nm is thus the optimum choice because here the pressure dependent spectral changes of the NADH absorption are negligible. In the case of the temperature dependence of the NADH absorbance (*e.g.* at 366 nm), the observed decrease follows from the superposition of the measured absorbance and the thermal expansion of the diluted aqueous solution. The intrinsic temperature effect on the optical density is obtained after correcting for the change in density. The result is shown in Fig. 2. It is obvious from the data given that the absorbance of NADH is affected by pressure, pH and temperature. Under

conditions of biologically significant pressures (≤ 1.2 kbar) the effects are comparatively small in the region of intense absorption at $\lambda \sim 366$ nm. They gain relative significance towards higher wavelengths.

Two contributions may be responsible for changes in optical density for nicotinamide adenine nucleotides: the intrinsic absorption of the nicotinamide and adenine part of the molecule, and intramolecular interactions of both chromophoric groups in the open and closed conformation of the molecule. The influence of pressure and temperature could be connected with either solvent effects or the equilibrium of the two conformers in solution [15]. High pressure is known to stabilize the association of aromatic bases [10]. On the other hand, increased temperature is expected to favour the open structure owing to the competition of the thermal energy

with the weak intermolecular interactions stabilizing the intramolecular stacking [16].

Significant shifts in the open \rightleftharpoons closed equilibrium would generate much more pronounced spectral changes than the ones observed in the present experiments. Without being able to exclude this explanation, the observed effects may be fully described by the previously mentioned dipole interactions between the aromatic chromophores and the solvent [12, 13].

Acknowledgements

This work was supported by grants of the Deutsche Forschungsgemeinschaft and the Fonds der Chemischen Industrie. G. S. was a recipient of a fellowship of the Friedrich Ebert Stiftung.

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