Biophysical Chemistry 21 (1985) 15–19
Elsevier

BPC 00892

THE INFLUENCE OF METHANOL ON THE INTERACTIONS OF CALCIUM WITH THE PYRIDINE NUCLEOTIDES

R. Kenneth BURKHARD

Department of Biochemistry, Kansas State University, Manhattan, KS 66506, U.S.A.

Received 21st March 1984
Accepted 6th July 1984

Key words: Calcium; Pyridine nucleotide; Interaction; Association constant; Enthalpy change; Calorimetry

The interactions of calcium with NAD^+, NADH, $NADP^+$ and NADPH in a 50% (by volume) methanol/water mixture (pH 7, 25°C) were studied by calorimetry. The association constants for 1 : 1 complex formation were found to be 6.6 ± 0.2, 270 ± 76, 18 ± 3 and 98 ± 10 for NAD^+, NADH, $NADP^+$ and NADPH, respectively. Comparing these to the association constants for an aqueous system reveals that as the polarity of the solvent system is decreased the interactions involving NAD^+, $NADP^+$ and NADPH are all decreased. In contrast, the interaction involving NADH is markedly increased. All the interactions were found to be endothermic.

1. Introduction

Interest in calcium is currently high even though its importance in biochemistry has long been known. One reason for this is the rather general role that calcium has in regulation. Because some of the enzyme systems that are regulated by calcium require pyridine nucleotides, a study was recently undertaken to determine the association constants for calcium with the pyridine nucleotides. From this it was found that in an aqueous system calcium forms rather loose complexes with the pyridine nucleotides with these interactions having association constants of approx. 20, 30, 90 and 200 for NAD^+, NADH, $NADP^+$ and NADPH, respectively [1].

In contrast to these results, about a decade ago Vinogradov et al. [2] found that calcium forms a rather tight complex with NADH in methanol and methanol/water systems. The association constant that can be calculated from their report is approx. 4×10^3. Thus, it appears that this interaction is greatly affected by the polarity of the system.

Living systems contain NAD^+, $NADP^+$ and NADPH as well as NADH and, even though the interactions of all of these with calcium have been studied in aqueous systems, only in the case of NADH has a study been made in systems of lower polarity. Because in vivo environments can be of low polarity, and because no study has been made to determine how interactions of the other pyridine nucleotides with calcium might be affected by changes in solvent polarity, it was felt that such a study was warranted. Accordingly, a study was made of the interactions of calcium with the pyridine nucleotides in methanol/water mixtures, the results of which show that lowering the polarity of the system markedly increases the extent of the interaction involving NADH, but decreases the interactions involving the other nucleotides.

2. Methods and materials

The interactions of calcium with the pyridine nucleotides were studied by calorimetry. This

Abbreviation: Pipes, piperazine-*N*,*N*′-bis(2-ethanesulfonic acid).

method differs from both of those used earlier [1,2]. The previous study in this laboratory used a calcium-sensitive electrode and the study performed by Vinogradov et al. used a fluorometric technique. However, calcium-sensitive electrodes cannot be used in nonaqueous systems and the fluorometric technique cannot be used on the oxidized forms of the pyridine nucleotides. Calorimetry, in contrast, provides a means of studying the interactions of both the oxidized and reduced forms of the pyridine nucleotides in nonaqueous systems.

A batch microcalorimeter similar to that described by Kitzinger and Benzinger [3] was used to measure the heat changes that occurred when 5 ml aliquots of calcium chloride and pyridine nucleotide solutions were mixed. For each nucleotide a series of experiments was performed keeping the total nucleotide concentration constant but varying the total calcium concentration. In all but a few experiments, the total nucleotide concentrations (after mixing in the calorimeter) were similar to those used in the earlier study performed in this laboratory involving an aqueous system ($\approx 1 \times 10^{-3}$ M) [1]. Again, in all but a few experiments the total calcium concentrations (after mixing in the calorimeter) ranged from approx. 0.01 to 0.1 M. These conditions were selected so that the corrected heat exchanges would be larger than 10 mcal and the highest and lowest extents of interaction for any given nucleotide would differ by about 30%. Experiments involving NAD^+ and $NADP^+$ were performed in duplicate using three different concentrations of calcium; those involving NADH and NADPH were also performed in duplicate but used seven and five different concentrations of calcium, respectively.

The water used for experimentation was first deionized, then boiled and cooled under nitrogen; the methanol was either Fisher Spectranalyzed or MCB Omnisolv grade methanol. The solvent system used in any given experiment was prepared in a quantity sufficient for preparation of all the solutions needed for that experiment. This was done to avoid heat changes that would arise if even slightly differing methanol/water mixtures were to be mixed in the calorimeter. The majority of the experimentation involved a solvent system consisting of a mixture of Tris and Tris-HCl in a 50% (v/v) mixture of methanol and water. The amounts of the reagents used to supply the buffer components of this solvent system were identical to those used to prepare the analogous aqueous system used in earlier studies [1] in which the pH was 7.0 and the ionic strength 0.1. The observed pH was always close to 7.0.

To determine possible proton exchange in the Ca-NAD^+ interaction a few experiments were performed in which the sodium salts of Pipes were substituted for Tris and Tris-HCl. As with the more commonly used Tris/Tris-HCl system, the observed pH values of the Pipes systems were always close to 7.0.

One of the problems encountered in this study was the limited solubility of the pyridine nucleotides in methanol. Because of this, a study was made to determine if solution would be complete in several methanol/water systems envisioned for experimentation. These studies involved application of the method of King [4] to determine the total soluble phosphate after filtration or centrifugation of possible experimental systems. The results from these studies then guided experimental design.

The pyridine nucleotide samples used were from lot 022088 Chromatopure NAD^+, lot 116500 Chromatopure NADH, lot 03900 $NADP^+$ and lots 116100 and 126100 NADPH as obtained from P-L Biochemicals, Inc. In a typical experiment a small sample of nucleotide ($\approx$ 30 mg) was quickly weighed and dissolved in the desired solvent system ($\approx$ 20 ml). The pH was quickly determined and adjusted, if necessary, to the value observed before addition of the pyridine nucleotide. No adjustments were made in the cases of NADH and NADPH, but small measured volumes of NaOH solutions ($\approx$ 50 μl) were added to the NAD^+ and $NADP^+$ systems. In these cases, appropriate additions of methanol were also made to keep the methanol/water ratio constant. The final volume was then adjusted to achieve solutions of approx. 2×10^{-3} M pyridine nucleotide. The NaOH solutions used for pH adjustment were prepared from a saturated solution of NaOH to minimize contamination due to carbonate. The amounts of enzymatically usable pyridine nucleotide were de-

termined by means of the alcohol dehydrogenase-ethanol, alcohol dehydrogenase-acetaldehyde, isocitrate dehydrogenase and NADPH-glutathione reductase methods [5]. These assays were done initially as close as practicable to the time of the calorimetric experiment and then at selected times extending over a 3 h period. This provided both purity and stability data which were used in subsequent calculations.

Calcium chloride solutions were prepared from reagent grade $CaCl_2 \cdot 2H_2O$. The concentrations of the stock calcium chloride solutions were determined by use of a Radiometer F2112Ca calcium-sensitive electrode after either making accurate 1 : 100 dilutions or reconstituting after low-temperature evaporation to dryness, using, in both cases, deionized water that had been boiled and cooled under nitrogen.

After correcting the observed heat exchanges for effects due to dilution, a nonlinear least-squares program was used to obtain the best fit for the following equation

$$q = \frac{q_{max} K [Ca_{free}]}{1 + K [Ca_{free}]}$$

where q is the corrected heat exchange, q_{max} the value for q if the extent of reaction were 100%, K the association constant for a 1 : 1 complex, and $[Ca_{free}]$ the concentration of the free Ca^{2+} at equilibrium. The computer program used for these calculations was based on one that had been developed and used by Delbert D. Mueller of this department to perform a similar type of calculation involving hydrogen-deuterium exchange in proteins [6]. Sillén [7–9] has developed similar procedures to analyze binding data. The estimates of q_{max} and K that were used to initiate the above calculations were obtained in the manner employed by Biltonen's group [10] in their study of the binding of cytidine monophosphate to ribonuclease. This involved iterative calculations to obtain the best fit for a double-reciprocal plot involving $1/q$ and $1/[Ca_{free}]$. This nonlinear approach has the advantage that all points are weighted equally and thus avoids the unequal weighting inherent in linear regression of double-reciprocal data.

3. Results and discussion

The association constants and enthalpy changes obtained from experiments involving calcium and the pyridine nucleotides in a 50% (v/v) methanol/water mixture are shown in table 1. It appears that differences exist among the pyridine nucleotides in their abilities to react with calcium in this type of system. This was not unexpected, since differences had been observed earlier for this type of interaction in an aqueous system [1]. However, the order of binding of calcium to the pyridine nucleotides is changed on going from an aqueous to a 50% methanol/water system. In water the order was $NAD^+ < NADH < NADP^+ < NADPH$; in 50% methanol/water it appears to be $NAD^+ < NADP^+ < NADPH < NADH$. Further, when one compares the association constant for any particular nucleotide in this methanol/water system to that for the water system, it is seen that the constant for the NADH system is markedly increased by raising the methanol concentration from 0 to 50%, but the constants for all the other systems are decreased. This is in accord with expectations because the calcium-NADH complex is the only one of the four with a net zero charge and its formation would be favored specifically by a decrease in solvent polarity.

The results obtained with the NADH system suggest that further increases in the concentration of methanol would result in further increases in the association constant for this interaction. This

Table 1

Association constants and enthalpy changes for calcium-pyridine nucleotide interactions in 50% (v/v) methanol/water mixtures (pH 7, 25°C)

The values shown below are based on the assumption that the impurities present in the pyridine nucleotides were chemically inert (e.g., water). See text for comments regarding the method of calculation and corrections for impurities.

Pyridine nucleotide	Association constant	Enthalpy change (kcal/mol)
NAD^+	6.6 ± 0.2	8.3 ± 0.2
NADH	270 ± 76	2.4 ± 0.1
$NADP^+$	18 ± 3	5.8 ± 0.4
NADPH	98 ± 10	3.4 ± 0.1

is undoubtedly true, since Vinogradov et al. [2] using a fluorometric technique to study the calcium-NADH interaction in 70–100% methanol have reported a dissociation constant of approx. 2.4×10^{-4} (the corresponding association constant is 4×10^{3}). It was hoped that calorimetric studies involving the NADH system could be extended to higher concentrations of methanol, but additional experiments toward this goal were disappointing. As mentioned above, the pyridine nucleotides exhibit limited solubilities in methanol. This presented a major problem for calorimetry. To maintain complete solution during calorimetry, the concentrations of reactants had to be decreased. Because of this, the observed heat exchanges became very low and the reactive errors quite large. The few data obtained, however, did suggest that the association constant for this interaction is considerably higher in 70% methanol than in 50% methanol. This would be expected from the observations made by Vinogradov et al. [2] and the suggestion that the interaction of calcium with NADH is quite sensitive to the solvent polarity with decreasing solvent polarity markedly increasing the extent of the interaction.

In a brief look at the enthalpy changes, all of the interactions appear to be endothermic. This was not unexpected since all of these interactions were endothermic in an aqueous system [1].

One of the problems encountered in working with the pyridine nucleotides is purity; another is stability. With the latter problem in view, experiments were designed to minimize error due to instability. Enzymatic assays of the pyridine nucleotide solutions over 3-h periods showed that the concentrations of NADH and NADPH solutions decreased slowly under the conditions of these studies (≈ 1 and 3% per h, respectively) whereas those of NAD^+ and $NADP^+$ did not. With this information experimentation was carried out, as far as practicable, so that the calorimetric portion of each experiment was performed at a specified time after dissolution of the nucleotide (usually ≈ 1.5 h). During this time interval, it was possible to make adjustments in pH and volume, load the calorimeter and establish a stable baseline on the recorder. The concentrations of NADH and NADPH at the times of the calorimetric experiments were then estimated by extrapolation of data from accompanying stability studies. When the problem of purity was investigated, the various pyridine nucleotide samples were found to be less than 100% pure. If the impurity in each case was water (or any other chemically inert substance), the observed purities could then be used without correction to calculate the desired association constants and enthalpy changes. This simple approach was used to obtain the values shown in table 1.

In the cases of the reduced nucleotides, it is reasonable that the impurities were the corresponding oxidized forms, and the decreases in the concentrations of the reduced forms resulted in concomitant increases in the oxidized forms. To determine how this possibility would affect the values shown in table 1 for NADH and NADPH in 50% methanol, additional calculations were performed using the association constants and enthalpy changes for NAD^+ and $NADP^+$ that are shown in table 1. These calculations were somewhat more complex than those outlined in section 2. They involved corrections for the heat exchanges and the decreases in free calcium that would be associated with the assumed concentrations of the oxidized pyridine nucleotides. The results of these calculations gave 359 and 2.3 kcal/mol as the association constant and enthalpy change, respectively, for NADH and 143 and 3.2 kcal/mol, respectively, for NADPH. Thus, while this type of correction would increase the calculated association constants for these two nucleotides, it would not alter the relative order of binding of the four nucleotides as a group. This type of correction would make the enthalpy changes only slightly smaller.

The uncertainties mentioned above suggest caution regarding numerical values that should be reported for these interactions. Even so, it appears, as mentioned earlier, that in 50% methanol the order of binding of calcium to the pyridine nucleotides is $NAD^+ < NADP^+ < NADPH <$ NADH, and that while the association constant for the calcium-NADH interaction is markedly increased by raising the methanol concentration from 0 to 50%, the constants for all the other interactions are decreased.

Comparison has already been made of the as-

sociation constants obtained in 50% methanol/water to those obtained in water. A few comments should be made regarding the enthalpy changes. Once again all these interactions are endothermic and thus are entropically driven. It appears, however, that certain of the interactions become more endothermic as the polarity of the solvent decreases. To determine if proton exchange with the buffer is a reasonable means of accounting for this, additional calorimetric experiments were performed with the NAD^+ system. This nucleotide was selected because it had the largest solvent-related increment in enthalpy change. Duplicate experiments at the highest calcium concentration gave mean heat uptakes of 33 mcal for a Tris-containing 50% methanol/water system and 34 mcal for a Pipes-containing system. Since the heats of protonation of Tris and Pipes are very different (-11.36 kcal/mol vs. -2.74 kcal/mol at 25°C and $\mu = 0.1$) [11], it was concluded that something other than proton exchange with the buffer was most likely responsible for the solvent-related increments in enthalpy changes that were noted. This agrees with the results for aqueous systems [1] in which little or no proton exchange was found to occur when calcium reacted with the pyridine nucleotides.

Finally, for each of the pyridine nucleotide pairs calcium reacts to a greater extent with the reduced form in both aqueous and 50% methanol/water systems. Because of this, increases in calcium would always be expected to increase the reduction potentials for the pyridine nucleotides. In aqueous systems, however, the effect of calcium would be relatively small because the ratio of either reduced association constant (K_r) to the corresponding oxidized association constant (K_0) is always approx. 2. In 50% methanol/water the effect of calcium on the $NADP^+$-NADPH system would also be small since K_r/K_0 is approx. 5. In contrast, the reduction potential for the NAD^+-NADH system would be more susceptible to changes in calcium in 50% methanol/water since K_r/K_0 is approx. 40. If one accepts a value of -0.032 V as the midpoint potential for the NAD^+-NADH couple in 50% methanol/water at 25°C, the association constants in table 1 suggest this potential does not change at 0.1 mM free calcium but rises to -0.029 and -0.016 V at 1 and 10 mM free calcium, respectively. Systems of lower polarity would undoubtedly exhibit more pronounced effects.

Acknowledgements

I wish to thank Dr. Delbert D. Mueller of this department for the use of his nonlinear least-squares program to evaluate my experimental data. This work was supported by the Kansas Agricultural Experiment Station.

References

1 R.K. Burkhard, Arch. Biochem. Biophys. 218 (1982) 207.
2 A. Vinogradov, A. Scarpa and B. Chance, Arch. Biochem. Biophys. 152 (1972) 646.
3 C. Kitzinger and T.H. Benzinger, Methods Biochem. Anal. 8 (1960) 309.
4 E.J. King, Biochem. J. 26 (1932) 292.
5 M.M. Ciotti and N.O. Kaplan, Methods. Enzymol. 3 (1957) 890.
6 P.A. McBride-Warren and D.D. Mueller, Biochemistry 11 (1972) 1785.
7 L.G. Sillén, Acta Chem. Scand. 16 (1962) 159.
8 L.G. Sillén, Acta Chem. Scand. 16 (1962) 173.
9 L.G. Sillén, Acta Chem. Scand. 18 (1964) 1085.
10 D.W. Bolen, M. Flögel and R. Biltonen, Biochemistry 10 (1971) 4136.
11 J.J. Christensen, L.D. Hansen and R.M. Izatt, Handbook of proton ionization heats (John Wiley, New York, 1976) p. 152, 159.