KINETIC ISOTOPE EFFECTS IN THE NAD- AND NADP-SPECIFIC ISOCITRATE DEHYDROGENASES OF PIG HEART

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1. Introduction

Pig heart muscle, as most mammalian tissues, contains two isocitrate dehydrogenases: an NADP-specific enzyme found both in the mitochondria and the cytoplasm and an NAD-dependent enzyme located in the mitochondria [1]. Both types of isocitrate dehydrogenase use the same side of the nicotinamide ring [2] and in both enzymatic reactions a proton from the solvent replaces the carboxyl group of isocitrate with retention of configuration [3–5]. Marked differences, however, have been noted in the catalytic properties and physical characteristics of these two enzymes. The NADP enzyme is relatively small, consisting of a single polypeptide chain with a mol. wt. of 58 000 and it is not generally classified as an allosteric enzyme [6]. In contrast, the NAD enzyme is considerably larger, with the catalytically functional species having a mol. wt. of 340 000 [7] and is composed of subunits of 40 000 mol. wt. [8]; this enzyme is activated by ADP and is considered as an important regulator of the Citric Acid Cycle [9]. This paper represents part of a continuing effort to elucidate the similarities and differences between a non-regulatory and allosteric enzyme in terms of catalytic mechanisms and chemical reactivity of functional groups.

The overall oxidative decarboxylation of isocitrate consists of an initial dehydrogenation to form oxalosuccinate, followed by the decarboxylation of a β-keto acid to yield α-ketoglutarate. For the NADP-specific enzyme, it has been shown that the slow process is the dehydrogenation reaction [10,11]. Two of the possible rate determining steps for the dehydrogenation reaction might be, 1) the transfer, of a proton from the hydroxyl group, or 2) the transfer of the hydride ion from the substrate to the coenzyme. Deuterium oxide solvent isotope effects have been used in biochemistry as well as physical organic chemistry to implicate proton transfer in the rate determining step of a reaction since such proton transfer reactions generally proceed more slowly in D_2O than H_2O as solvent [12, 13]. It was shown previously that the maximum velocity of the reaction catalyzed by the NADP-dependent isocitrate dehydrogenase is 4.9 times greater when measured in H_2O than in D_2O suggesting that general base catalysis might be involved in the slow step of that enzymatic reaction [10]. The present paper compares the solvent isotope effect for the NAD-specific enzyme. Furthermore, the possibility that hydride transfer might be rate determining is examined for both enzymes using isocitrate specifically deuterated in the C-2 position.

2. Materials and methods

The NAD-dependent isocitrate dehydrogenase was purified from pig heart by chromatography on DEAE-cellulose and cellulose phosphate followed by gel filtration on Sepharose 6B, as described by Shen et al. [8]. The enzyme was dialyzed exhaustively against 0.05 M PIPES buffer, pH 7.0, containing 20% glycerol and 0.5 mM $MnSO_4$ for purposes of stability and stored at 85°C prior to use. The NADP-specific isocitrate dehydrogenase of pig heart was obtained from Boehringer as a special preparation free of albumin, and was purified to homogeneity by procedures reported previously [14]. Deuterium oxide (99.8%) was

purchased from Stohler Isotope Chemical Company, while the sodium borodeuteride was supplied by Merck, Sharp and Dohme. Barium oxalosuccinate was either synthesized by acid hydrolysis of triethyloxalosuccinate (K and K Laboratories) by the procedure of Ochoa [15] or was purchased from Sigma Chemical Co.; solutions of sodium oxalosuccinate were prepared immediately before use. Coenzymes and substrates were obtained from the Sigma Chemical Company.

Isocitrate deuterated at the C-2 position was prepared by treating oxalosuccinate with sodium borodeuteride, a reagent that selectively reduces carbonyl groups in the presence of carboxylate groups [16]. The sodium borodeuteride was added at 0°C (to yield a final concentration of 0.06M) to a solution of sodium oxalosuccinate (0.024 M) in 0.04 M imidazole chloride buffer, pH 6.0. This pH was selected as optimal, since the rate of decarboxylation of oxalosuccinate increases as the pH is raised above 6, and the rate of decomposition of sodium borodeuteride is enhanced as the pH is reduced [17]. The amount of threo-D_S-isocitrate formed was determined enzymatically using NADP-dependent isocitrate dehydrogenase as described below. Under these conditions, approximately 15–20% of the oxalosuccinate was converted to threo-D_S-isocitrate. Purification was achieved by applying the reaction mixture to a Dowex-1-formate column, washing with water and then eluting the isocitrate with 3 N formic acid, in accordance with LaNoue et al. [18]. Fractions containing the isocitrate peak were detected, after neutralization, by enzymatic assay and these were evaporated to dryness. The nmr spectrum of the deuterated product, measured at a concentration of 0.4 M in D_2O, exhibited the multiplets expected for the H–C–COO^- and CH_2 protons, but lacked the characteristic doublet of the H–C–OD proton [19]. The absence of any signal for a C-2 proton strongly indicates that the incorporation of deuterium at this position is essentially complete.

The initial velocity of isocitrate dehydrogenase-catalyzed reactions were measured at 25°C by the increase in absorbance at 340 nm, using a Gilford Model 240 spectrophotometer equipped with an expanded scale recorder (0.1 A full scale). In determining the pH dependence of the NAD-specific isocitrate dehydrogenase, imidazole–0.072 M chloride and sodium–0.072 M acetate buffers were used with 1.33 mM, 20.0 mM manganous sulfate, DL-isocitrate and NAD, respectively, in a total volume of 1.0 ml. The pH of the reaction mixture was measured immediately after determination of the velocity. Doubling the concentrations of these substrates had no significant effect on the rates. For the deuterium oxide experiments all substrates and buffers were prepared in D_2O and the deuteron content was calculated from the relationship, $pD = pH_{measured} + 0.40$ [20,21]. The reaction was initiated by the addition of 0.01 ml of enzyme in a water buffer in order to minimize the exchange of deuterium into the protein and thus the deuterium oxide reaction solutions actually contained about 1% H_2O.

In the measurements of the Michaelis constants for isocitrate-1-[^{1}H] and isocitrate-2[^{2}H], the concentrations are given for threo-D_S-isocitrate, which is known to be the only stereoisomer which functions as the substrate for these isocitrate dehydrogenases. The solvent used was H_2O. In the case of the NAD enzyme, the isocitrate concentration was varied while the total $MnSO_4$ and NAD concentrations were maintained constant at 1.25 mM and 1.00 mM, respectively, and the buffer used was imidazole–0.012 M chloride, pH 5.97. For the NADP enzyme, the total $MnSO_4$ and NADP concentrations were kept constant at 1.25 mM and 0.106 mM, respectively, and the buffers used were imidazole–0.36 M chloride, pH 5.97, triethanolamine–0.036 M chloride, pH 7.04 and triethanolamine–0.036 M chloride, pH 8.07.

3. Results and discussion

3.1. Velocities with isocitrate-2-[^{2}H] or isocitrate-2-[^{1}H] as substrate

It has previously been demonstrated that the hydrogen at the C-2 position of isocitrate is transferred by NADP- and NAD-dependent isocitrate dehydrogenases to the A-side of the nicotinamide ring of NADP or NAD [2]. Isocitrate specifically deuterated at the C-2 position is now used as a substrate for both isocitrate dehydrogenases in order to measure the kinetic isotope effect. In the case of the NAD-specific isocitrate dehydrogenase the K_m for total isocitrate is known to increase approximately 100-fold as the pH is raised from 6 to 8 which led to the proposal that a dibasic form of isocitrate is the actual substrate for the enzyme [22]. Fig. 1 shows Lineweaver-Burke plots

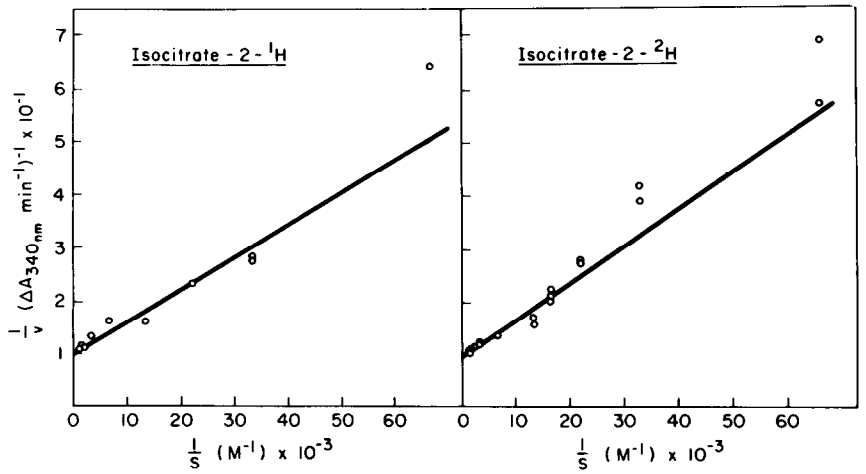

Fig. 1. K_m for isocitrate-2-[^{1}H] and isocitrate-1-[^{2}H] as substrate for NAD-specific isocitrate dehydrogenase.

for isocitrate-2-[^{1}H] and isocitrate-2-[^{2}H] as substrates for the NAD enzyme at pH 5.97; the K_m values are calculated as 59 and 71 μM, respectively. The extrapolated values of maximum velocity are essentially the same for the two substrates (table 1), indicating that hydride transfer from isocitrate cannot be the rate limiting step.

Table 1 shows that in the case of the NADP-dependent isocitrate dehydrogenase the K_m for total isocitrate does not vary significantly over the pH range from 6 to 8. This observation is consistent with a tribasic form of isocitrate being the actual substrate of the NADP enzyme. The K_m for deuterated isocitrate at pH 7.0 is somewhat lower than for the corresponding hydrogen-containing isocitrate. The maximum velocity at pH 7.04, obtained either by extrapolation of the Lineweaver-Burke plot or by measurement of the rate at an isocitrate concentration that is more than 50 times higher than the K_m, is only 1.15 times greater with isocitrate-2-[^{1}H] than isocitrate-2-[^{2}H]. Similarly, the maximum velocities measured at pH 6 and 8 exhibit isotope effects of approximately 1.2–1.3. It has been suggested that kinetic deuterium isotope effects of 2–15 provide evidence for cleavage of a

Table 1

V_{max} and K_m-isocitrate for NAD- and NADP-specific isocitrate dehydrogenase using isocitrate-2-[^{1}H] and isocitrate-2-[^{2}H]

Enzyme	pH	K_m (Isocitrate-2-[^{1}H])	K_m (Isocitrate-2-[^{2}H])	V^*_{max}-isocitrate-2-[^{1}H] / V_{max}-isocitrate-2-[^{2}H]
		(μM)	(μM)	
NADP-isocitrate dehydrogenase	5.99	2.8	–	1.26
	7.04	3.0	2.0	1.15
	8.07	2.7	–	1.30
NAD-isocitrate dehydrogenase	5.97	59	71	0.95

* For the NAD-specific isocitrate dehydrogenase at pH 5.97 and the NADP-specific isocitrate dehydrogenase at pH 7.04, values of V_{max} for isocitrate-2-[^{1}H] and isocitrate-2-[^{2}H] are extrapolated from the corresponding Lineweaver-Burke plots. For the NADP-specific isocitrate dehydrogenase at pH 5.99 and 8.07, the values given for V_{max} were those measured at the relatively high threo-D$_S$-isocitrate concentration of 162 μM.

carbon–hydrogen bond in the rate limiting step of a reaction [23]. The magnitude of the isotope effect observed for NADP-specific isocitrate dehydrogenase is too low to support such a postulate.

3.2. Deuterium oxide solvent isotope effect

The maximum velocity of the reaction catalyzed by the NAD-specific isocitrate dehydrogenase was measured in water and in deuterium oxide over a range of pH (or pD) from approximately 5–7 in order to distinguish between an effect of the solvent on the pK of an essential ionizable group, and an effect on the intrinsic pH-independent maximum velocity. Fig. 2 shows that the pK of the ionizable group in the enzyme-substrate is not appreciably different in D_2O as compared to H_2O. In contrast, the intrinsic maximum velocity is 5.2 times greater in water than in deuterium oxide as solvent. Solvent isotope effects in biochemical systems must be interpreted with caution [24]; it is possible, for example, than an apparent change in V_{max} is only a reflection of a solvent-induced change in the Michaelis constants, or in the conformation of the enzyme resulting from exchange of hydrogen for deuterium with the protein. However, the substrate concentrations used are all high relative to their respective Michaelis constants [22], and increasing the concentrations in either water or deuterium oxide did not increase the measured velocities. The enzyme is added to the assay solutions in water, and is only exposed to deuterium oxide for a period of approximately one min while the catalytic rate is being measured; this time period is short relative to the intervals generally used to obtain significant deuterium–hydrogen exchange into the structure-determining groups which participate in stable internal hydrogen bonding in protein [25]. The simplest interpretation of these results is that a proton transfer is involved in the rate-determining step of the oxidative decarboxylation catalyzed by NAD-specific isocitrate dehydrogenase. A quantitatively similar solvent isotope effect ($V_{max\text{-}H_2O}/V_{max\text{-}D_2O}$= 4.9) has been reported for the reaction catalyzed by NADP-dependent isocitrate dehydrogenase which has been localized to the step involving dehydrogenation of isocitrate, rather than the subsequent oxalosuccinate decarboxylation step [10]. The kinetic isotope data for the two isocitrate dehydrogenases suggest similar mechanisms for the allosteric and non-regulatory enzymes. An amino acid residue of each enzyme may function as a general base to abstract a proton from the C-2 hydroxyl group of isocitrate in the rate limiting step of the reaction.

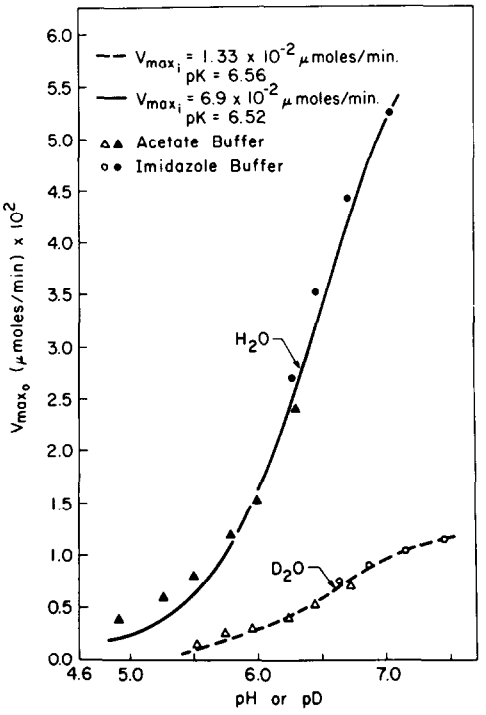

Fig. 2. pH dependence of V_{max} for the NAD-dependent isocitrate reaction in H_2O and D_2O. The points are experimental and the lines are theoretical, calculated from the equation $V_{max_o} = [V_{max_i}]/[1 + (H^+/K)]$ where V_{max_o} = observed maximum velocity at a given (H^+); V_{max_i} = intrinsic maximum velocity; and K = dissociation constant of activity-dependent ionizable group in the enzyme–substrate complex.

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