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^{1}H NMR OF SUCCINATE BINDING TO ASPARTATE TRANSCARBAMYLASE A COMPARISON OF RESULTS IN D_2O AND H_2O

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The interaction of succinate with aspartate transcarbamylase from Escherichia coli has been studied by magnetic resonance relaxation measurements of the dicarboxylic acid methylene protons in H_2O solutions. The pH and temperature dependence of the relaxation in the presence of either native aspartate transcarbamylase or its catalytic subunit in H_2O solutions is qualitatively very similar to the corresponding situation utilizing D_2O as the solvent. From previous results of measurements in D_2O [C.B. Beard and P.G. Schmidt, Biochemistry 12 (1973) 2255] a mechanism was proposed involving 2 protonated groups affecting succinate binding and titratable over the pH range 7–10. Quantitatively, fitting the data from H_2O solutions to the mechanism yields values of the fitting parameters generally in good agreement with the D_2O experiments.

The main exceptions are the pK_a values calculated for the two titratable groups. For these species the values obtained in the presence of the catalytic subunit are 6.7 and 7.8 in H_2O solutions versus 7.3 and 8.6 in D_2O solutions. In the presence of native enzyme the corresponding values are 6.8 and 8.3 in H_2O versus 7.6 and 9.2 in D_2O. These observed differences are consistent with differences in ionization constants of weak acids in D_2O relative to H_2O. The results imply that succinate interaction with the enzyme active site is similar in the two solvents.

1. Introduction

Proton magnetic resonance (PMR) relaxation studies of biologically interesting systems are often carried out in D_2O solutions, as experimental restrictions may preclude the use of H_2O as a solvent. The effects of this solvent substitution are difficult to predict, but may include alterations of kinetic and structural properties. One can at the very least expect changes in the ionization constants of titratable amino acid side chain groups [1]. This uncertainty of solvent effect makes direct comparison of PMR data obtained in D_2O solutions with other data from H_2O solutions a risky procedure. These concerns motivated the present study of the pH and temperature dependences of the PMR relaxation of succinate binding to aspartate transcarbamylase (C_6R_6) and the aspartate transcarbamylase catalytic subunit (C_3) in H_2O solutions. Since similar experiments employing D_2O as the solvent have been reported [2,3] the effect of the solvent can be determined.

2. Materials and methods

Native aspartate transcarbamylase and its catalytic subunit were obtained by the method of Gerhart and Holoubek [4]. Enzyme concentrations were determined from the absorption at 280 nm and by the radioactive assay of Porter et al. [5].

Samples for PMR experiments were prepared by combining appropriate volumes of four solutions: (1) the appropriate enzyme species in a borate-imidazole-glycylglycine buffer, pH 7.9; (2) borate-imidazole-glycylglycine buffers of varying pH, (3) unbuffered succinate solutions of varying pH, (4) solutions of dilithium carbamyl phosphate in borate-imidazole-glycylglycine buffers of varying pH. These last solutions were prepared immediately prior to use to minimize the decomposition of the carbamyl phosphate. Sodium hydroxide and acetic acid were used to adjust

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the pH of the stock solutions. The final solutions were approximately 3×10^{-4} M in enzyme catalytic sites; 0.04 M or 0.05 M in succinate, 0.004 M in carbamyl phosphate, 0.015 M in imidazole, 0.015 M in borate, 0.005 M in glycylglycine. 2-Mercaptoethanol was present at 2×10^{-3} M to stabilize the enzyme, and EDTA was present at 2×10^{-4} M to protect against heavy metal ion contamination. The samples also contained 0.02 M t-butanol which served as a linewidth standard in the relaxation measurements. All chemicals were commercial preparations and were used without further purification. Glass distilled H_2O was used exclusively as the solvent.

The pH values of the samples were determined both before and after the PMR experiments. These values were found to differ by as much as 0.2 to 0.3 pH units for the highest pH samples due to decomposition of carbamyl phosphate. This decomposition was minimized by keeping the samples in ice between measurements. Whenever the initial and final pH values differed the values were averaged and this average is the value reported.

Linewidths of the succinate methylene resonance were measured at 100 MHz on a Varian HA100 spectrometer using the solvent H_2O as the internal lock signal. The succinate resonance is approximately 2.5 ppm upfield of the H_2O signal and can be observed without interference from the very strong lock signal. The temperature of the spectrometer probe was controlled by the standard Varian temperature control unit and was determined before and after each relaxation measurment by direct placement of a thermocouple into the probe.

3. Results and treatment of data

3.1. pH and temperature dependence of succinate relaxation in the presence of C_3

The linewidth of the succinate methylene resonance was measured for seven C_3 samples in the pH range of 6.9–9.4 at five temperatures between 14 and 40°C. The linewidth of internal t-butanol was subtracted in each case to correct for line broadening due to magnetic field inhomogeneity. The transverse relaxation rate, T_2^{-1}, was calculated from the relation: $T_2^{-1} = \pi\Delta\nu'$ where $\Delta\nu'$ is the difference between the succinate linewidth and that of the dilute t-butanol in the sample. The results are plotted in fig. 1 as T_2^{-1} versus temperature. The lowest pH sample shows an increase in relaxation rate with increasing temperature. Relaxation rates of samples of intermediate pH increase with temperature, reach a maximum, and then decrease, while the highest pH samples exhibit a monotonic decrease in relaxation rate with increasing temperature. The results from Beard and Schmidt [2] obtained in D_2O solutions are reproduced in fig. 2. Comparison of figs. 1 and 2 reveals a strong qualitative resemblance between the H_2O and D_2O results with the D_2O data shifted somewhat to higher pH.

3.2. pH and temperature dependence of succinate relaxation in the presence of C_6R_6

Relaxation rates were determined in the same manner as above for six C_6R_6 samples in the pH range 7.2–9.7. Each sample was measured at five temperatures between 14 and 39°C. The results are presented in fig. 3, while fig. 4 from ref. [3] shows the corresponding results in D_2O. Again the results in H_2O and D_2O are quite similar and retain a slight shift of the D_2O data to higher pH.

3.3. Analysis of the PMR relaxation data

Hammes et al. [6] found from temperature-jump measurements that in the presence of bound carbamyl phosphate the binding of succinate to C_3 could be described by a two step process:

$$E + I \underset{k_{-1}}{\overset{k_1}{\rightleftharpoons}} EI \underset{k_{-2}}{\overset{k_2}{\rightleftharpoons}} EI', \qquad (1)$$

where succinate (I) binds in a rapid step to form EI followed by a slow isomerization to give EI'. A subsequent study by PMR of the pH dependence of succinate to C_3 [2] elaborated on eq. (1) to include protonation steps:

$$\begin{array}{ccccc} E & \overset{H^+}{\rightleftharpoons} & EH & \overset{H^+}{\rightleftharpoons} & EH_2 \\ & & k_1 I \uparrow\downarrow k_{-1} & & I \uparrow\downarrow \\ & & EHI & \overset{H^+}{\rightleftharpoons} & EH_2I \underset{k_{-2}}{\overset{k_2}{\rightleftharpoons}} (EH_2I)'. \end{array} \qquad (2)$$

Two protein groups involved in succinate binding are titrated over the pH range 7 to 10. A higher pK_a group

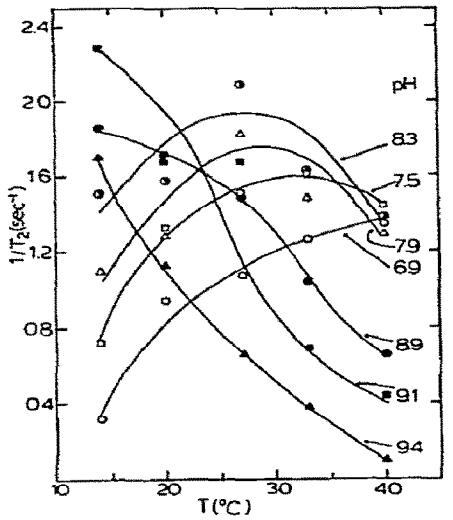

Fig. 1. Transverse relaxation rate of succinate protons in the presence of the ATCase catalytic subunit and solvent H_2O as a function of temperature and pH. $1/T_2$ values: (○), at pH 6.9; (□), at pH 7.5; (△), at pH 7.9; (●), at pH 8.3; (■), at pH 8.9; (▲), at pH 9.1; (▲), at pH 9.4. Samples were 0.05 M in succinate, 3.5×10^{-4} M in catalytic sites. $1/T_2$ values are $\pm 0.2\ s^{-1}$.

must be protonated in order for succinate to bind to the enzyme (EHI) and a lower pK_a moiety when protonated promotes the slow isomerization step to form $(EH_2I)'$. Eq. (2) implies a four site exchange problem with possible succinate environments: EHI, EH_2I, $(EH_2I)'$, and free I. It was found, however, that the equilibrium between EH_2I and $(EH_2I)'$ greatly favors the latter conformation leading to only a small fraction of bound succinate in the EH_2I form. In addition EH_2I appears similar to the EHI species in terms of the relaxation rate of succinate nuclei [2]. Thus, succinate relaxation resulting from the mechanism of eq. (2) can more simply be described by eq. (3):

$$EH + I \rightleftharpoons EHI \overset{H^+}{\rightleftharpoons} (EH_2I)'. \qquad (3)$$

Eq. (3) represents a 3 site exchange situation for succinate with the concentration of the small molecule much greater in one site (unbound) than in the other two. Such a case was dealt with by Swift and Connick [7], who solved for the transverse relaxation rate $1/T_2$ of nuclei on molecules undergoing chemical exchange among magnetically non-equivalent sites.

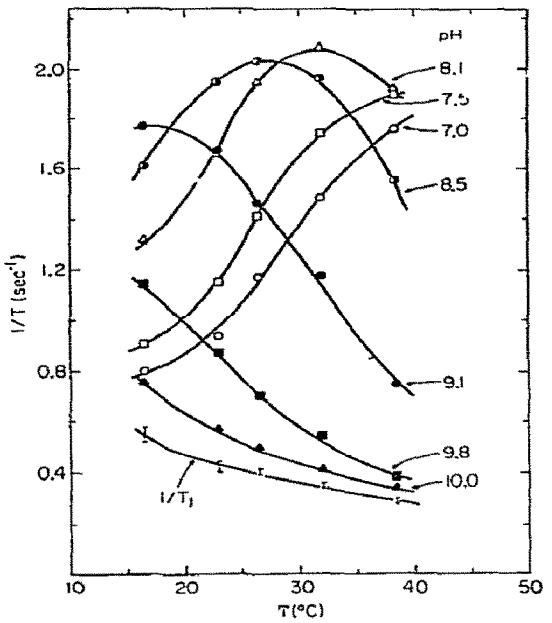

Fig. 2. Relaxation rates of succinate protons in the presence of the ATCase catalytic subunit and solvent D_2O as a function of temperature and pH. $1/T_2$ values: (○), at pH 7.0; (□), at pH 7.5; (△), at pH 8.1; (●), at pH 8.5; (●), at pH 9.1; (■), at pH 9.8; (▲), at pH 10.0. All pH values are uncorrected meter readings. Samples were 0.05 M in succinate and 1.7×10^{-4} M in catalytic sites.

With the conditions that exchange of succinate is rapid from the EHI complex and slow between the EHI and $(EH_2I)'$ species (compared to relaxation rate and chemical shift differences between the sites) the observed relaxation rate of protons on succinate in the presence of aspartate transcarbamylase is given by [2]

$$1/T_2 - 1/T_{2,I} = \mathcal{P}_{EHI} P_0 [1/T_{2,EHI}] + \mathcal{P}_{(EH_2I)'} P_0/\tau_{(EH_2I)'}, \qquad (4)$$

where P_0 is the total fraction of succinate bound to the enzyme, $\mathcal{P}_{EHI}$ and $\mathcal{P}_{(EH_2I)'}$ represent the fraction of bound succinate which is in the EHI (or EH_2I) and $(EH_2I)'$ forms, respectively, $T_{2,EHI}$ is the transverse relaxation time of succinate protons in the EHI complex, $\tau_{(EH_2I)'}$ is the lifetime of the conformational isomer $(EH_2I)'$, and $1/T_{2,I}$ is the transverse relaxation rate of free succinate.

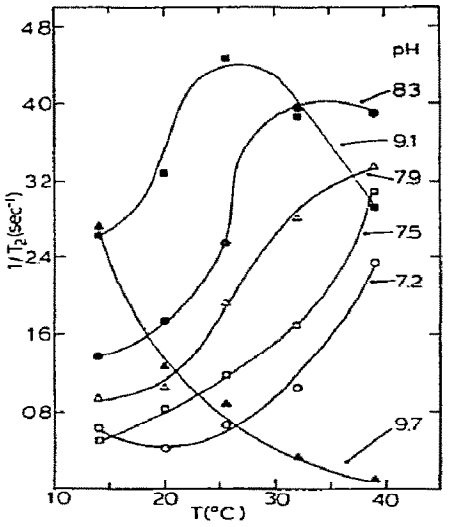

Fig. 3. Transverse relaxation rate of succinate protons in the presence of native ATCase and solvent H_2O as a function of temperature and pH. $1/T_2$ values: (○), at pH 7.2; (□), at pH 7.5; (△), at pH 7.9; (●), at pH 8.3; (■), at pH 9.1; (▲), at pH 9.7. Samples were 0.04 M in succinate and 3.0×10^{-4} M in catalytic sites. $1/T_2$ values are ± 0.2 s^{-1}.

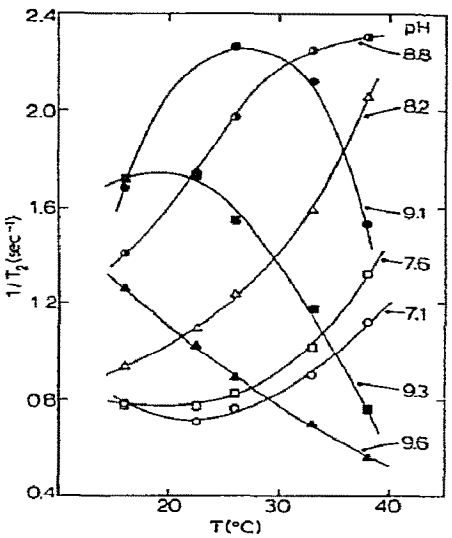

Fig. 4. Transverse relaxation rate of succinate protons in the presence of native ATCase and solvent D_2O as a function of temperature and pH. $1/T_2$ values: (○), at pH 7.1; (□), at pH 7.6; (△), at pH 8.2; (◇), at pH 8.8; (●), at pH 9.1; (■), at pH 9.3; (▲), at pH 9.6. All pH values are uncorrected meter readings. Samples were 0.04 M in succinate and 2×10^{-4} M in catalytic sites.

The relaxation data of figs. 1—4 can be readily understood in a qualitative sense through eq. (4). At low pH the $(EH_2I)'$ species predominates and proton relaxation is determined by the slow exchange rate of succinate from that complex; $1/T_2$ increases with increasing temperature. At higher pH values the fraction of bound succinate in EHI, $\mathcal{P}_{EHI}$, increases at the expense of $\mathcal{P}_{(EH_2I)'}$. The measured relaxation rate increases because $1/T_{2,EHI}$ is significantly larger than $1/\tau_{(EH_2I)'}$ over most of the temperature range. At the highest pH values succinate no longer binds to the active site at all ($P_0 \rightarrow 0$) due to titration of the EH form of the enzyme. Thus the nuclear relaxation rate decreases toward zero at the highest pH values.

In the present study succinate proton linewidth data ($1/T_2$) for the enzyme plus carbamyl phosphate in H_2O were fit in a manner similar to the previous data in D_2O. Three important distinctions arise for the D_2O and H_2O results. In D_2O, T_2 was measured by a transient technique, adiabatic half passage or T_1 in the rotating frame [8]. This method is quite sensitive, and gives accurate, precise results. In addition, T_1, the spin—lattice relaxation time, is easily measured in a variation of the method. In water the presence of the large H_2O resonance precludes use of the transient method and necessitates use of slow passage techniques. Our choice for T_2 determinations was linewidth measurements whose accuracy and precision are both significantly inferior to the transient technique.

Secondly, the analysis of the D_2O relaxation results took advantage of the T_1 measurements to use the difference $1/T_2 - 1/T_1$ in place of $1/T_2 - 1/T_{2,I}$ in eq. (4). The former expression is advantageous in that it provides a correction to the latter due to the difference in viscosity of the enzyme solution as compared with the solution with enzyme absent [9]. However, in the D_2O experiments $1/T_1$ was found to be small and virtually independent of temperature and pH. Thus the absence of data for $1/T_1$ in the H_2O experiments introduces no significant error.

Finally, the PMR experiments utilizing D_2O as solvent were performed at 220 MHz while those in H_2O performed at 100 MHz. It was found, however, that the succinate relaxation in D_2O was independent of frequency between 100 MHz and 220 MHz [2,3]. The comparisons discussed here then are unaffected by this difference in spectrometer frequency.

3.4. Data fitting procedure

In practice the fitting procedure requires expressions for the several equilibrium constants implicit in eq. (2);

$$K_{a2} = [E]\,[H^+]/[EH], \quad (5)$$

$$K_{EHI} = [EH]\,[I]/[EHI], \quad (6)$$

$$K_{EH_2I} = [EH_2]\,[I]/[EH_2I], \quad (7)$$

$$K_{a1} = [EHI]\,[H^+]/[EH_2I], \quad (8)$$

$$K_{con} = [EH_2I]/[(EH_2I)'] . \quad (9)$$

Beard and Schmidt [2] have shown that the fraction of enzyme in any form represented in eq. (2) is related to these equilibrium constants in a simple manner.

$$\mathcal{P}_E = K_{a1}K_{a2}K_{EHI}K_{con}/D, \quad (10)$$

$$\mathcal{P}_{EH} = K_{EHI}K_{a1}K_{con}[H^+]/D, \quad (11)$$

$$\mathcal{P}_{EHI} = K_{a1}K_{con}[H^+]\,[I]/D, \quad (12)$$

$$\mathcal{P}_{EH_2I} = K_{con}[H^+]^2\,[I]/D, \quad (13)$$

$$\mathcal{P}_{(EH_2I)'} = [H^+]^2[I]/D, \quad (14)$$

where

$$D = K_{a1}K_{a2}K_{EHI}K_{con} + K_{EHI}K_{a1}K_{con}[H^+] + K_{a1}K_{con}(H^+)(I) + (K_{con}+1)[H^+]^2[I]. \quad (15)$$

The fitting procedure involves estimating, on the basis of the experimental relaxation times obtained, each of the two relaxation terms in eq. (4) assuming the logarithm of each term varies linearly with the inverse of the temperature. Initial values of pK_{a1}, pK_{a2}, pK_{EHI}, pK_{con} and the enthalpy changes associated with each of these steps, ΔH_1, ΔH_2, ΔH_{EHI}, ΔH_{con} are also estimated. The fractional enzyme populations are then calculated from eqs. (10)–(14) and a value for $1/T_2 - 1/T_{2,I}$ is obtained from eq. (4). Twelve parameters, the 4 pK's and the 4 enthalpy changes described above as well as the slopes and intercepts of the log (relaxation term) versus inverse temperature plots for each term of eq. (4), are then iterated until the set of values is found which yields the best fit of calculated to experimental values for $1/T_2 - 1/T_{2,I}$.

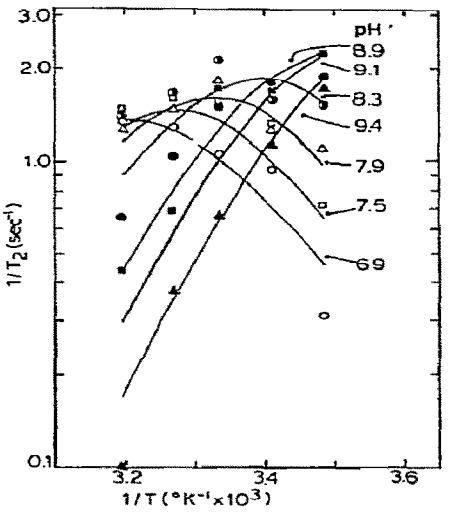

Fig. 5. Logarithm of the succinate transverse relaxation rate as a function of reciprocal temperature, experimental (points) and calculated (solid curves), in the presence of H_2O and ATCase catalytic subunit. Conditions are as in fig. 1.

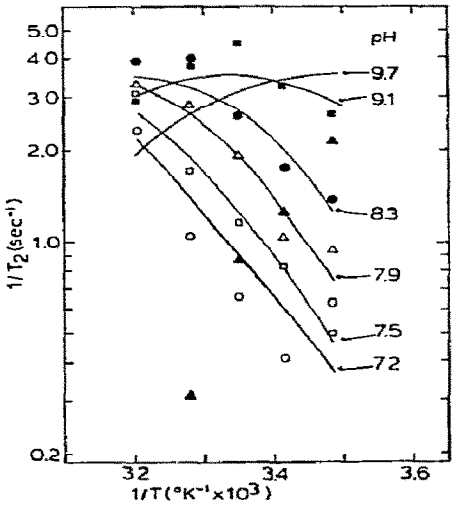

Fig. 6. Logarithm of the succinate transverse relaxation rate as a function of reciprocal temperature, experimental (points) and calculated (solid curves), in the presence of H_2O and native ATCase.

Table 1
Comparison of the best fit parameters from relaxation data in H_2O to that in D_2O at 28°C

	C_3		C_6R_6	
	H_2O	D_2O [a]	H_2O	D_2O [a]
pK_{a1}	6.7	7.3	6.8	7.6
pK_{a2}	7.8	8.6 [b]	8.3	9.2
pK_{con}	0.9	1.1	1.1	1.6
pK_{EHI}	2.6	2.1 [b]	3.5	1.8
ΔH_1	12	12	10	16
ΔH_2	7	[c]	9	2
ΔH_{con}	16	14	14	12
ΔH_{EHI}	8	[c]	10	8

[a] Acid dissociation constants based on pD = meter reading + 0.4.
[b] Only a sum determined directly. Individual values calculated using results of temperature-jump study [6].
[c] Only the sum $\Delta H_2 + \Delta H_{EHI}$ = 12 kcal/mole was obtained.

The quality of the fit was particularly sensitive to the values for pK's. Variation of more than about 0.1 units from the best fit values significantly deteriorated the quality of the fit. It was possible, however, to vary pK's *pairwise* by up to 0.2 units each without greatly affecting the fit as long as the sums $pK_{a1} + pK_{con}$ and $pK_{a2} + pK_{EHI}$ remained constant. The enthalpies exhibited similar behavior, it being possible to vary each by as much as 3 kcal/mole as long as the sums $\Delta H_1 + \Delta H_{con}$ and $\Delta H_2 + \Delta H_{EHI}$ remained constant. The values of 0.2 units and 3 kcal/mole then serve as error limits of the best fit parameters. The results are shown in figs. 5 and 6 for C_3 and C_6R_6, respectively. In both figures the smooth curves represent the calculated values while the experimental values are indicated by the points. The best fit values for the pK's and enthalpies are presented in table 1 for $C_3$and C_6R_6. The corresponding values found in the D_2O experiments are also given.

4. Discussion

4.1. Relationship of linewidths to $1/T_2 - 1/T_{2,I}$

As noted previously, $1/T_2$ was calculated from the difference in linewidth of succinate and t-butanol which, due to its negligible natural linewidth, served as an indicator of the inhomogeneity of the magnetic field. Succinate free in solution also has a long T_2 (~ 3 s) and negligible natural linewidth. Thus, subtraction of the t-butanol linewidth is, within the error of the measurement, equivalent to subtracting both the magnetic field inhomogeneity and the free succinate contribution. Thus the relaxation rate calculated from $\pi\Delta\nu'$ is equivalent to $1/T_2 - 1/T_{2,I}$.

4.2. Comparison of results with C_3 in H_2O to D_2O

4.2.1. Ionization constants

The best values of the fitting parameters are shown in table 1 which consists of values from both this study and the previous D_2O experiments [2,3]. In the D_2O work pK_a values were reported based on uncorrected pH meter readings for the meter standardized with H_2O buffers. Therefore, we have added 0.4 pH units to the acid ionization constant pK values in table 1 to correct for the deuterium isotope effect on a glass electrode [10]. With this correction pK_{a1} is 0.6 pH units greater in D_2O and pK_{a2} is 0.8 pH units greater. In a study of weak acid ionization constants Bunton and Shiner [11] found an increase of 0.5 to 0.7 pH units for pK's of the acids in D_2O relative to H_2O. Our results are consistent with a simple deuterium isotope effect.

The PMR results are also in accord with the findings of Stark [1] that C_3 exhibited very similar activity versus pH profiles in H_2O and D_2O. The approximate bell-shaped pH versus activity profile was shifted 0.8 pH units higher in D_2O but retained the same shape and same maximum activity value. An important point emerges from both the NMR and enzyme activity measurements. The change in pK for both titratable groups is approximately the same on going from H_2O to D_2O. This implies that the mechanism of catalysis and binding of substrate inhibitors should be similar in the two solvents, validating comparisons of measurements in D_2O and H_2O.

4.2.2. Enthalpies

Values for ΔH_1 and ΔH_{con} are similar for H_2O and D_2O. In the D_2O study [2] individual values for ΔH_2 and ΔH_{EHI} were not obtained but, rather, their sum was used as a fitting parameter. This yielded a best fit value of 12 kcal per mole, within the experimental error limits the same as the sum of ΔH_2 and ΔH_{EHI} in the present study, 15 kcal.

4.2.3. Rate constants

The PMR relaxation rates at low pH are dominated by the slow exchange between EH_2I and $(EH_2I)'$. A value of $100\ s^{-1}$ for $1/\tau_{(EH_2I)'}$ (which is equivalent to k_{-2} of eq. (2)) at 28° is obtained from the best fit of the data for C_3 in H_2O. The forward rate constant, k_2, is then obtained from $K_{con} = k_{-2}/k_2$ which gives $k_2 = 7.9 \times 10^2\ s^{-1}$. These values can be compared to the rate constants $k_{-2} = 7.5 \times 10^2\ s^{-1}$ and $k_2 = 3.2 \times 10^3\ s^{-1}$ obtained from experiments in D_2O [2]. Both rate constants are significantly lower in H_2O which may point to an isotope effect in the slow enzyme-succinate isomerization step. But the nature of this step is poorly understood and it is not yet possible to assign specific roles for labile protons in the process.

4.3. Comparison of results with C_6R_6 in H_2O to D_2O

4.3.1. Ionization constants

As with C_3, the pH and temperature dependence of succinate in the presence of C_6R_6 in H_2O is qualitatively similar to that in D_2O as evidenced by figs. 3 and 4. Quantitatively, the pK values in table 1 show a relationship similar to that exhibited with C_3 *albeit* one in which the difference due to solvent is somewhat greater. Nonetheless, ionization constant differences in H_2O and D_2O can account for these changes in pK_{a1} and pK_{a2} within the estimated error of the best fit values. The correspondences between the values of pK_{EHI} and between those of pK_{con} are not as good. The comparison, indicated in fig. 6, of experimental points with calculated values reveals a poor fit at the highest pH sample. This same difficulty was encountered in the D_2O study [3] despite several attempts at refinement of the fitting procedure.

At high pH the binding of succinate becomes sufficiently weak that even at a concentration of 0.05 M it no longer saturates its binding sites. Allosteric effects become important and lead to behavior which is not accounted for by eq. (2).

4.3.2. Enthalpies

As is apparent from table 1 the values for ΔH_1 and ΔH_2 in H_2O do not closely resemble those in D_2O even after allowance is made for the rather large error limits. The values obtained in the present study do, however,seem more reasonable for single ionization constants. For example, if ΔH_1 refers to the ionization of the imidazole group of a histidine residue (vide infra) it would be expected to be 7–8 kcal/mole [12].

4.3.3. Rate constants

As noted previously, k_{-2} of eq. (2) can be determined from the best fit value of $\tau_{(EH_2I)'}$. For C_6R_6 experiments in H_2O solutions a value of $32\ s^{-1}$ is obtained for k_{-2}. Although this is virtually identical to the value obtained in D_2O, $33\ s^{-1}$ [1], one must be cautious in drawing conclusions as the fit in the present study was not greatly sensitive to $\tau_{(EH_2I)'}$ over a range corresponding to $k_{-2} \approx 10$–$35\ s^{-1}$.

The rate constant k_2 was obtained as before yielding $4 \times 10^2\ s^{-1}$ in H_2O solutions versus $1.3 \times 10^3\ s^{-1}$ in D_2O solutions [2]. The differences here are due, of course, to the difference in K_{con} between the two solvents.

4.4. Correlation times for macromolecular motion

The transverse relaxation rate of succinate bound to the EHI enzyme species is related to the rotational correlation time, τ_R, of the bound succinate by [13]:

$$\frac{1}{T_{2,EHI}} = \frac{3}{40} \sum_{i,j} \frac{\hbar^2\gamma^4}{r_{ij}^6}\left(6\tau_R + \frac{10\tau_R}{1+(\omega_0\tau_R)^2} + \frac{4\tau_R}{1+4(\omega_0\tau_R)^2}\right), \quad (16)$$

where $\hbar$ is Planck's constant divided by 2π, γ is the proton magnetogyric ratio, ω_0 is the proton resonance frequency in rads per second and r_{ij} is the internuclear distance between protons on the succinate molecule. For succinate binding with its carboxyls in the *cis* orientation [5] evaluation of eq. (16) yields values for τ_R of 3×10^{-8} s when bound to C_3 and 5×10^{-8} s when bound to C_6R_6. This provides only fair agreement with the values derived from the experiments in D_2O: 7×10^{-8} s with C_3 and 10×10^{-8} s with C_6R_6. Part of this discrepancy is due to the difference in viscosity between H_2O and D_2O, the viscosity of D_2O being approximately 20% greater than that of H_2O in the temperature range studied [14]. According to the Stokes–Einstein model, the rotational correlation time varies directly with viscosity. Also, although no pH dependence of $1/T_{2,EHI}$ (and

thus of τ_R) has been considered, it is reasonable to assume that the enzyme structure is sensitive to change in pH; i.e., that there exists more than one form of EHI species, which may differ enough in tertiary structure to significantly affect τ_R.

4.5. Comparison of NMR result with other studies

Porter et al. [5] in steady state kinetics experiments of the pH dependence of succinate inhibition of C_3, found evidence of a group with a pK_a of 7.1, presumably a histidine residue, at the active site. Vanaman and Stark [15] studied the pH dependence of the inactivation of C_3 by 5,5'-dithiobis-(2-nitrobenzoate) (DTNB) and observed a dependence of the inactivation on a group with a pK_a of 7.9. Although DTNB inactivates by reaction with sulfhydryl groups on the enzyme, the group with pK_a of 7.9 is not likely to be a sulfhydryl itself, based on studies of C_3 preparations containing several different chemically modified sulfhydryl groups [16]. The pK_{a1} and pK_{a2} values for C_3 observed in the present study agree quite well with the above results although there is no assurance that they are measures of the same ionizations.

For the most part, the results of our PMR experiments in H_2O agree well with those done in D_2O and do not contradict any conclusions of previous studies. This correspondence is, a priori, neither obvious nor general and thus, it is important that it be ascertained that the use of D_2O as a solvent imposes no great perturbation on the mechanism of enzyme action if PMR work done in D_2O is to be compared to other studies in which the solvent is H_2O.

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