Effect of Allosteric Ligands on the Mechanism and Stability of Nicotinamide–Adenine Dinucleotide Specific Isocitrate Dehydrogenase from Yeast†

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ABSTRACT: Ligand-dependent changes of the yeast NAD-specific isocitrate dehydrogenase reaction mechanism are suggested from kinetic isotope effects of *threo*-D_S-[2-^{3}H]isocitrate and from a comparison of the activation energies at various substrate and modifier concentrations. At low NAD and isocitrate concentrations (isotope effect ≈ 4) the reaction is compatible with a random-order, rapid-equilibrium mechanism and a rate-limiting hydrogen transfer, but at 1 mM NAD or in the presence of 0.2 mM 5′-AMP (isotope effect ≤ 2.5) the mechanism converts to a partially ordered type. Sharp breaks in Arrhenius plots and discontinuities of the kinetic isotope effects indicate a conformational transition at 21° in presence of less than 0.2 mM NAD. However, >1 mM NAD or 5′-AMP stabilize the high-temperature conformation between 35 and 10°. Below the transition temperatures the enzyme is still active and fully reversible to the high-temperature state. The cold sensitivity is shared with other oligomeric regulatory enzymes. The refined kinetic tests revealed different kinetic properties and changes of the mechanism for an enzyme classified as a K-type enzyme (Monod *et al.* (1965), *J. Mol. Biol. 12*, 88). It is suggested that the study of more examples of regulatory "K enzymes" will lead to a revision of the former definition specifying that alterations at the binding site will also lead to different kinetic properties.

Nicotinamide–adenine dinucleotide dependent isocitrate dehydrogenase from yeast seems to share with other regulatory enzymes the property that substrates and modifiers strongly interact decreasing the substrate concentrations required for half-maximal velocity. All changes of reactivity could be attributed to modifications of dissociation constants with the maximal velocity remaining unchanged (Hathaway and Atkinson, 1963; Atkinson *et al.*, 1965). Allosteric enzymes which exhibit differential affinities of ligands toward different enzyme states without changes of intrinsic rate constants were classified by Monod *et al.* (1965) as K systems.

Models which have been proposed to rationalize K systems differ mainly in the modes of interaction between substrates, modifiers and the protein and in the number of preexisting or newly formed enzyme conformations (Atkinson, 1966; Koshland and Neet, 1968). The symmetry model (Monod *et al.*, 1965) explains the propagation of affinity changes by influencing the equilibrium of preexisting conformations; it does not consider whether the different conformations exhibit different kinetic behavior. On the other hand, the induced-fit model (Koshland and Neet, 1968) predicts a series of conformational species with different kinetic and binding properties. In this case it is more difficult to understand how pure K systems can be realized. These authors suggested that the modifiers might pull conformational equilibria in the direction of stabilized enzyme forms. Increasing consideration was given recently to models explaining sigmoidal substrate concentration dependence by ligand–ligand interactions between closely connected binding sites or even overlapping subsites for bidentate ligands (Fischer *et al.*, 1970). With this model, changes in K_m without any consequent alteration in V_{max} might be explained.

In view of the reasonable assumption that alterations at the binding site leading to changes in ligand affinity might also change the reactivity of the substrates (although these have rarely been seen so far), we looked for kinetic methods which would indicate subtle changes of rate constants in K systems under conditions of different substrate or modifier concentrations.

Sigmoidal initial velocity *vs.* concentration data are difficult to interpret for the evaluation of the rate-determining step if it is to be assumed that the rate constants are themselves dependent on the functional state of the enzyme at different concentrations. Therefore a concentration-independent kinetic parameter for the evaluation of rate constant changes was needed. In cases where an isotope substituted bond is broken in the rate-determining step, the competitive measurement of the isotope discrimination in the reaction products provides a substrate concentration-independent determination of the rate-determining step (Simon and Palm, 1966).

We also studied the effect of other conditions to detect changes in kinetic constants. It can be shown that the Arrhenius plots between 20 and 35° of the apparent V_{max} with isocitrate as variable substrate are different at low as compared to high NAD concentrations or with or without 5′-AMP. This supports the conclusions from the kinetic isotope effect studies. The nonlinear Arrhenius plots obtained with isocitrate dehydrogenase in a more extended temperature interval are not uncommon with regulatory enzymes which undergo multisite and subunit interactions (*cf.* Kastenschmidt *et al.*, 1968; Gregolin *et al.*, 1968; Irias *et al.*, 1969) and are indicative of conformational changes. Massey *et al.* (1966) have pointed out that such conformational changes could alter the reaction mechanism. This was indeed the case with isocitrate dehydrogenase.

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Methods

Reagents. 5′-AMP, coenzymes, lactate dehydrogenase, NADP-dependent isocitrate dehydrogenase, and alkaline phosphatase were purchased from Boehringer Mannheim. Other reagents used were: *threo*-D_S-isocitrate (Roth, Karlsruhe), Dowex 1-X8, DEAE-cellulose, CM-cellulose (Serva, Heidelberg), D-[1-^{3}H]glucose (The Radiochemical Centre, Amersham), bakers' yeast (Hefe-Mayer, München), Sephadex G-25, Sephadex G-200, Dextran 250 ($\bar{M}_W \approx 250{,}000$) (Deutsche Pharmacia GmbH, Frankfurt).

threo-D_S-[2-^3H]*Isocitrate.* The stereospecifically labeled compound was prepared according to Chen and Plaut (1963) by reversal of the NADP-dependent reaction of isocitrate: NADPH + H^+ + CO_2 + α-ketoglutarate $\rightleftharpoons$ isocitrate + $NADP^+$. Instead of [^{3}H]NADPH, we used the coupled-enzyme reaction

$$\text{[1-}^3\text{H]glucose 6-phosphate} + \text{NADP}^+ \overset{\text{G6PDH}}{\rightleftharpoons} \text{6-phosphogluconolactone} + \text{NADPH} + \text{H}^+$$

to provide labeled NADPH. If small concentrations of NADP are used in the NADPH-regenerating system [*A*,*B*-4-^{3}H]-NADPH is formed, but only the A-specific hydrogen is transfered to *threo*-D_S-isocitrate (*cf.* Wenzel and Brühmüller, 1967). In a small tapered test tube were placed 400 μmoles of Tris-acetate buffer, 2 μmoles of [1-^{3}H]glucose 6-phosphate (specific radioactivity 7.6×10^9 dpm/μmole), 38 μmoles of glucose 6-phosphate, 32 μmoles of $MnSO_4$, 60 μmoles of α-ketoglutarate, 0.5 μmole of NADP, 2.0 ml of 0.1 M $NaHCO_3$ saturated with CO_2, 11 units of glucose-6-phosphate dehydrogenase, and 0.6 unit of NADP-linked isocitrate dehydrogenase; final volume 8.6 ml, pH 7.5. The closed tube was kept in an atmosphere of CO_2. After denaturation in a boiling-water bath enough 1 M NaOH was added to adjust the pH to 9.7. Unreacted glucose 6-phosphate was destroyed by addition of 6 μmoles of $ZnSO_4$ and 15 units of alkaline phosphatase. After 1-hr incubation the solution was placed on a 2.2×21 cm Dowex 1-X8 (200–400 mesh, formate form) column. After addition of 200 ml of water, a linear gradient elution (150 ml of water + 150 ml of formic acid) was employed (Busch and Hurlbert, 1952). The pooled labeled fractions were freed from formic acid in a Büchi evaporator, and delactonized by addition of a slight excess 1 M NaOH and heating for 10 min at 85°. The product was free from glucose 6-phosphate and α-ketoglutarate. The tritium label is quantitatively transferable to NADP in the presence of NADP-linked isocitrate dehydrogenase. The recovery was 5.3 μmoles, 5.9×10^7 dpm/μmole.

Enzyme Assay. The yeast isocitrate dehydrogenase assay was performed in a final volume of 1 ml with the following concentrations: 1 mM D,L-isocitrate, 33 mM $MgCl_2$, 0.2 mM 5′-AMP, and 0.03 M Tris·HCl (pH 7.5). The reaction was followed at 366 nm. One enzyme unit corresponds to the reduction of 1 μmole of NAD/min at 25°.

Protein Determination. Protein in crude preparations was determined by the biuret method (Beisenherz *et al.*, 1953). In highly purified preparations the method of Lowry (Lowry *et al.*, 1951) was used.

Purification of Yeast Isocitrate Dehydrogenase. The procedure of Hathaway and Atkinson (1963) was modified to yield more stable preparations of the highly purified enzyme. Commercial bakers' yeast was suspended in 0.05 M phosphate buffer (pH 5.6) and aerated with O_2 for 24 hr. The cells were disrupted in a Biox-X press (AB-Biox, Sweden) at −15°. All further steps until the repeated precipitation with $(NH_4)_2SO_4$ follow the original procedure. The ammonium sulfate precipitate was suspended in 10 ml of 0.05 M phosphate buffer (pH 7.0) and stored with little loss of activity at −15°. Further purification was performed only prior to use with 1-ml aliquots of the stable stock solution as the highly purified preparations are less stable.

A 1-ml aliquot of the preceding suspension was placed on Sephadex G-25 according to Hathaway and Atkinson (1963). The active fraction of the eluate was placed on a 1.6×11.5 cm CM-cellulose column, previously equilibrated with 5 mM potassium phosphate, 16 mM KCl, 1 mM EDTA, and 0.5 ml/l. of mercaptoethanol (pH 7.0). The enzyme was then quantitatively eluted with the same buffer. This was followed by chromatography on a 1×15 cm DEAE-cellulose column, previously equilibrated with 0.01 M sodium phosphate (pH 7.5) and 0.01 M sodium phosphate, 0.06% 2-mercaptoethanol, and 0.5% Dextran 250 (pH 7.5). All subsequent buffers were supplemented by the same concentrations of 2-mercaptoethanol and Dextran 250. Elution was performed with 25 ml of 0.02 M sodium phosphate followed by a linear gradient of 50 ml of water/50 ml of 0.15 M sodium phosphate (pH 7.0). Fractions of 1 ml were collected. The first isocitrate dehydrogenase containing fraction had the highest specific activity. It is free from other NAD-reducing or NADH-oxidizing enzymes. The purification is summarized in Table I. After storage of the enzyme for 24 hr at 23°, a decrease of not more than 25% of enzyme activity was observed. If the CM-cellulose step was omitted, a considerably less stable enzyme preparation was obtained (*cf.* Hathaway and Atkinson, 1963).

Molecular Weight. A partially purified enzyme preparation (stock solution after $(NH_4)_2SO_4$ precipitation) was applied to a 2.85×60 cm Sephadex G-200 column at 4° and chromatographed in ascending mode. In the absence of 5′-AMP a value of 236,000 ± 2000 was estimated using known protein standards (Figure 1). The error of the method is given as 10% by Andrews (1964, 1965).

Kinetic Measurements; Isotope Effect Determination. All rate measurements were followed spectrophotometrically in a thermostated Eppendorf photometer at 366 nm, ϵ_{NADH} = 3.3 cm^2/μmole. A standard assay contains in 3–6 ml: 0.15–1.5 mM *threo*-D_S-isocitrate, 0.16–2.3 mM NAD, 1.6 mM $MgCl_2$, 0.033 M Tris·HCl, and 0.05–0.1 IU of isocitrate dehydrogenase. [2-^{3}H]Isocitrate was used as substrate in isotope effect determinations. The molar specific activity of the accumulated NADH (R_{bt}) at a spectrophotometrically measured percentage of reaction (f) was compared with the molar specific activity of the product at 100% reaction ($R_{b\infty}$) using the following formula to calculate the isotope effect

$$\frac{k_H}{k_{^3H}} = \frac{\ln (1 - f)}{\ln \left(1 - \frac{R_{bt}}{R_{b\infty}} f\right)}$$

Nomograms of this function are available (Collins, 1964).

Usually the reaction was allowed to proceed to about 10–20% of completion. If the reaction proceeds slowly, immediate transfer to a DEAE-cellulose column in the cold (see temperature dependence) is sufficient to stop the reaction after the percentage of the reaction is measured. The content of the cuvette is placed on a 1×8 cm DEAE-cellulose column (phosphate form, water washed). The elution of NADH by a linear gradient (100 ml of H_2O/100 ml of 0.15 M sodium phosphate, pH 8.0) was monitored by 260-nm absorption. NADH

TABLE I: Summary of the Purification of NAD-Specific Isocitrate Dehydrogenase from Bakers' Yeast.

Fraction	Vol (ml)	Act. (Units)	Sp Act. (Units/mg of Protein)	Yield (%)
Crude extract	40	26	0.02	
Second $(NH_4)_2SO_4$ precipitate dissolved in 0.05 M phosphate buffer (pH 7.0)	10	19.2	0.1	73.8
Sephadex G-25, eluate[a]	9.3	1.9	0.11	73
CM-cellulose, eluate	14	1.7	0.125	65.4
DEAE-cellulose eluates	1	0.6	12.0	23.1
	4	0.9		34.6

[a] 1-ml aliquots (10%) of the preceding step.

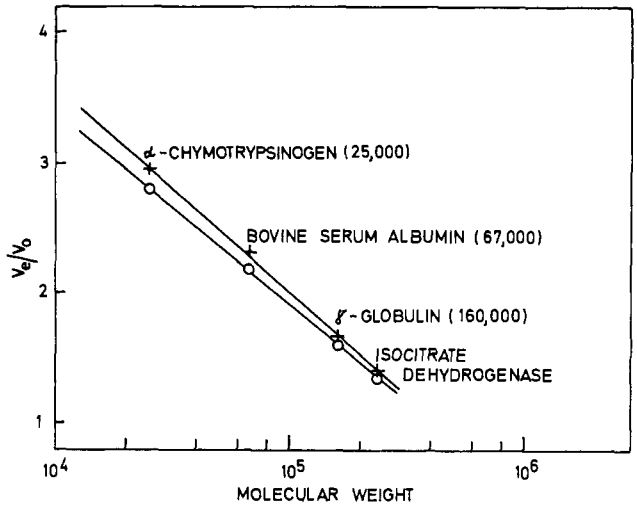
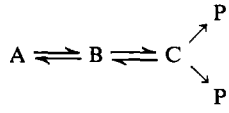


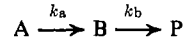
FIGURE 1: The molecular weight of isocitrate dehydrogenase in the absence of 5'-AMP. Protein standards are plotted (O) before and (+) after chromatography of the isocitrate dehydrogenase. V_e effluent volume, V_0 void volume.

fractions were analyzed quantitatively at 334 nm and by the disappearance of NADH in presence of lactate dehydrogenase and pyruvate at 334 nm (Klingenberg, 1962). Aliquots (0.5 ml) in 15 ml of Bray solution (Bray, 1960) were counted in a Packard Tri-Carb, Model 3314. Inhomogeneous counting samples in the presence of phosphate buffer were cleared by addition of 0.5 ml of phosphoric acid (85%). Average counting efficiency was 7%. Internal standardization was done with [^{3}H]toluene and used to convert counts per minute into disintegrations per minute.

Studies of Enzyme Mechanisms by Kinetic Isotope Effects. Kinetic studies are intended to clarify the minimal requirements, necessary for a hypothetical mechanism. Besides the well-known criteria for enzyme kinetics, such as the Haldane relationship, Dalziel (1957) relationship or product inhibition patterns (Cleland, 1963), kinetic isotope effects proved themselves especially valuable for characterizing mechanisms of dehydrogenases (Simon and Palm, 1966). For instance, primary isotope effects of tritium-labeled substrates as large as 2.5–5 could be correlated with the rate-limiting hydrogen transfer in yeast alcohol dehydrogenase which obeys a random-order, rapid-equilibrium mechanism (Palm, 1966). Smaller secondary isotope effects of 1.3–1.4, are observed for the same substrates in the liver alcohol dehydrogenase reaction (Palm *et al.*, 1968a) where product dissociation is rate limiting (Theorell–Chance mechanism). Secondary isotope effects are defined by altered force constants or steric requirements for the isotopic bond in the rate-limiting step (Halevi, 1964; Simon and Palm, 1966). In order to characterize changes in the kinetic parameters of the same enzyme following variations of the experimental conditions, kinetic isotope effects allow for the following conclusions. (1) Isotope effects determined by competitive methods are independent on substrate concentration. This is the case with ^{3}H substrates which are usually present together with an excess of unlabeled molecules. One of the most important advantages of measuring isotope effects is that it can dispense with extrapolation to small or infinite substrate concentrations for the evaluation of defined kinetic constants. This is a special advantage in cases of nonlinear substrate dependence of allosteric enzymes. Therefore a change of the isotope effect is indicative for a change of one or more kinetic steps. (2) Up to now, the possibility of interpreting the varying conditions leading to either the observation of the undiminished isotope effect of the rate-limiting step or to negligible isotope effects if no isotopic bond is changed in the rate-limiting step was not sufficiently explored. For the purpose of analyzing such influences, we simulated part of the general dehydrogenase mechanism (Colowick *et al.*, 1966) as a computer model. Several sets of substrate concentration (A) and rate constants were tested in the model



under the assumption, that one or more of the intermediate steps would have an isotope effect on the rate constant, leading to an observable isotope discrimination in the product P. It was found that for consecutive steps of the type



the observable isotope discrimination in the product P, caused by an isotope effect of k_a, becomes lower as the ratio k_b/k_a drops. From conventional kinetics, a relationship $k_b < k_a$ is synonymous with k_b rate limiting. Even if k_b is ten times smaller than k_a a sizeable isotope discrimination should be observed in the products, but its magnitude might be very similar to a secondary isotope effect. Thus, a small reduced primary isotope effect in a dehydrogenase reaction can arise from a slow step following hydrogen transfer. The source of secondary isotope effects in the dehydrogenase mechanism was found in several cases also in a slow step after the hydrogen transfer (Simon and Palm, 1966; Palm, 1968). In any event, in both cases one can safely assume that small isotope effects are indicative for a slow step behind the hydrogen-transfer step. (3). From a chemical point of view kinetic isotope effects allow one to delineate the transition state coordinates. For the biochemist kinetic isotope effects are of interest because he may see how the protein participates in the

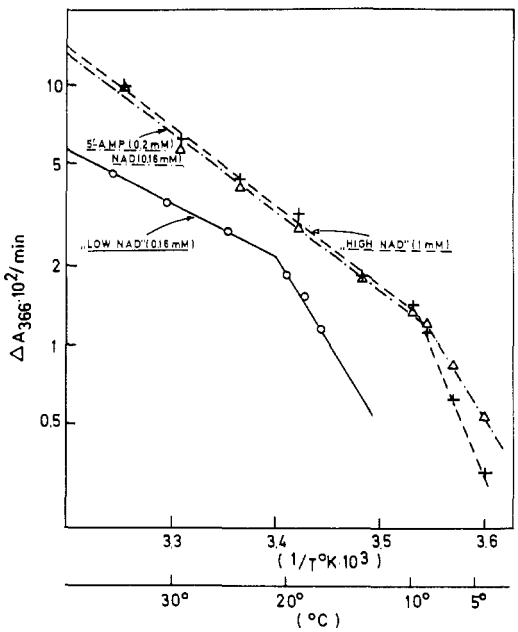


FIGURE 2: Arrhenius plots of isocitrate dehydrogenase activity. Log V_{max} is plotted *vs.* $1/T$ °K. The reaction mixture contained (in 33 mM Tris·HCl buffer (pH 7.5 at 25°)): (○) 0.16 mM NAD, 0.15–1.5 mM isocitrate, and 1.6 mM $MgCl_2$. Only the plot for 1.5 mM isocitrate is drawn, as identical slopes were observed at other isocitrate concentrations. (+) 1 mM NAD, 0.16 mM isocitrate, and 1.6 mM MgCl. (△) 0.16–1 mM NAD, 0.16–0.8 mM isocitrate, 0.2 mM 5′-AMP, and 1.6 mM $MgCl_2$; only the plot for 0.16 mM isocitrate is drawn. Higher concentrations of substrates were used with no influence on the slopes until substrate inhibition was observable. All values are corrected for temperature-dependent pH changes of Tris buffer.

formation of the transition state complex. According to theories of Westheimer (1961) and Bigeleisen (1964) the isotope effect decreases when the transition states assume a product-like arrangement. This might be achieved in enzyme catalysis by a favorable arrangement of substrates and it might be further supported by conformational changes in the protein. We will consider conformational influences on the isotope effect in the discussion.

Results and Discussion

General Properties of the Enzyme Preparation. Partial refinements in the preparative purification of yeast isocitrate dehydrogenase are detailed in Methods. The properties of this enzyme preparation were kinetically identical with the best preparations used by Atkinson (Hathaway and Atkinson, 1963; Atkinson *et al.*, 1965). We observed approach to maximal velocity in the absence of 5′-AMP at 0.2 mM NAD and 0.8 mM isocitrate (0.7 mM reported). Little or no reaction was observed below an isocitrate concentration of 0.15 mM under these conditions. In the presence of 0.2 mM 5′-AMP, V_{max} is approached at 0.2 mM isocitrate. An identical V_{max} is obtained in the presence of 1–1.2 mM NAD and no further influence of 5′-AMP can be seen. The enzyme becomes inhibited by high isocitrate concentrations (3 mM) in the absence of 5′-AMP.

Temperature-Dependent Changes of Activation Energy and Conformation. The temperature dependence of apparent V_{max} was studied over the range 5–40°. Precautions had to be taken in order to get reliable estimates for V_{max} excluding changes caused by lowered substrate affinity due to temperature dependence of K values. The difficulties arise from the sigmoidal rate *vs.* substrate concentration curve, which excludes a linear extrapolation to infinite substrate concentration. At high concentration of NAD or in the presence of 5′-AMP the possibility that further addition of substrate could lead to a further increase in velocity was carefully eliminated. At low concentrations of NAD, we plotted log v_i at isocitrate concentrations of 0.17, 0.42, 0.66 and 1.5 mM *vs.* $1/T$ °K. In each case we found identical slopes (Figure 2) from which activation energies were calculated (Table II).

TABLE II: Activation Energies and Transition Temperatures.

Conditions[a]	Transition Temp (°C)	Activn Energy: Upper Branch (cal/Mole)	Activn Energy: Lower Branch (cal/Mole)
1.5 mM Isocitrate–0.16 mM NAD	20.8	9,200	29,700
0.16–0.8 M Isocitrate–1.0 mM NAD	9.7	13,800	44,600
0.2 mM 5′-AMP–0.16 mM isocitrate–0.16 mM NAD	8.8	13,800	30,000
0.2 mM 5′-AMP–0.8 mM isocitrate–0.16 mM NAD	9.5	13,200	33,000
0.2 mM 5′-AMP–0.8 mM isocitrate–1.0 mM NAD	9.2	14,000	39,400

[a] Further details, see Figure 2.

From the data the following are concluded. (1) At room temperature, the activation energy in the absence of 5′-AMP and at low NAD concentrations is 9200 cal/mole. At high concentrations of NAD (1 mM) an increase of the slope corresponding to 13,800 cal/mole was observed. A similar increase of the slope was observed by addition of 5′-AMP at low NAD concentrations. The activation energy was not changed significantly if both substrates were present at saturating concentrations. The major effect of 5′-AMP on the activation energy might therefore be exerted *via* its effect on the apparent NAD saturation. (2) All log $V_{max}/(1/T)$ plots have a break, thus suggesting a different mechanism at lower temperature, possibly due to a cold induced conformational change. (3) High concentrations of NAD or 5′-AMP prevent a transition to the less active conformation at temperatures above 10°.

All temperature-dependent changes of velocity were fully reversible. Therefore even partial irreversible denaturation cannot account for the much higher activation energy at low temperatures. No lag phase was observed if the reaction of a sample was started by addition of 5′-AMP.

Isotope Effects at Different Ligand Concentrations. threo-D_s-[2-^{3}H]Isocitrate reacts with yeast isocitrate dehydrogenase under various conditions at a remarkably slower rate than the unlabeled substrate (Table III). The magnitude of the kinetic isotope effects points to significant primary isotope effects, which are usually between 3–5 in other enzyme reactions with tritium-labeled substrates (Simon and Palm, 1966).

TABLE III: Isotope Effects in the Reaction of *threo*-D_s-[2-^{3}H]-Isocitrate with Isocitrate Dehydrogenase of Yeast.[a]

Isocitrate (mM)	NAD (mM)	5′-AMP (mM)	Temp (°C)	Isotope Effect
		pH 7.5		
0.15	0.16		25.0	3.95 ± 0.2
1.0	0.5		25.0	2.90
1.0	1.0		25.0	3.03
0.15	0.83		22.0	1.86
0.15	2.3		22.0	1.67
0.15	1.0		6.5[b]	9.9
0.15	0.16	0.2	25.0	2.51 ± 0.02
1.0	0.33	0.2	25.0	2.48
		pH 6.5		
0.15	0.16		25.0	5.17 ± 0.09
0.15	0.16	0.2	25.0	5.66 ± 0.05

[a] All incubations were performed in 33 mM Tris·HCl, 1.6 mM $MgCl_2$, and additions and pH stated in the table. The isotope effects are corrected for the fraction of reaction (Collins, 1964). [b] pH 8.0.

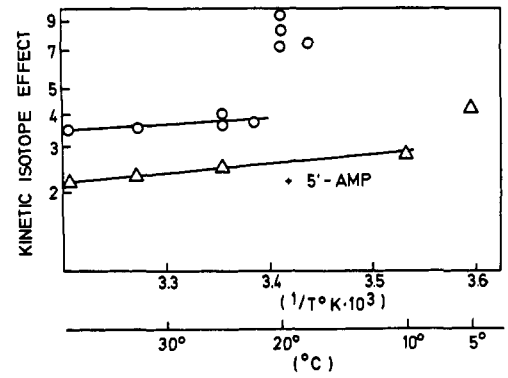


FIGURE 3: Temperature dependence of kinetic isotope effects. The log of the kinetic isotope effect is plotted *vs.* $1/T$ °K. Conditions: (○) 0.16 mM NAD, 0.15 mM *threo*-D_s-[2-^{3}H]isocitrate, 1.6 mM $MgCl_2$, and 33 mM Tris·HCl (pH 7.5 at 25°). (△) 0.2 mM 5′-AMP added.

To assess the gradual changes of the isotope effect the kinetic model presented in the theoretical section was used. A large primary isotope effect, as found at low NAD and isocitrate concentrations ($k_H/k_{^3H}$ = 4), is generally considered to be due to a rate-limiting hydrogen transfer. The unchanged magnitude of this primary effect will be observed in the specific radioactivity of the products (see Methods) only if consecutive steps behind the hydrogen transfer are considerably faster. It is reasonable to propose that ten times faster consecutive steps will not diminish the observable isotope effect within the limits of experimental error. If, as in the present study, the observable isotope effect drops from 4 to 3, only 2.5 times faster rates of consecutive steps are compatible with the decreased isotope effect. Similarily an isotope effect of 2.5 corresponds to equal rates of hydrogen transfer and consecutive steps, and considerably smaller rate constants for consecutive steps obtain with the smaller isotope effects observed in presence of high concentrations of NAD.

Temperature Dependence of Isotope Effects. The temperature dependence of isotope effects was studied under conditions similar to the evaluation of activation energies. Theoretical considerations predict a linear decrease of log isotope effect *vs.* $1/T$ °K (Wolfsberg and Stern, 1964). At higher temperatures, the temperature dependence at low NAD concentrations and in presence of 5′-AMP is as theoretically predicted (Figure 3). At low NAD and isocitrate concentrations a sharp break occurs at 20.9° with extraordinarily high isotope effects below this temperature. In the presence of high concentrations of NAD or 0.2 mM 5′-AMP the isotope effects also increase continuously as the temperature is lowered down to 10° and much higher isotope effects occur below this temperature (Figure 3 and Table III). Because exceedingly high enzyme and substrate concentrations are required to study isotope effects at the slow velocities of the enzyme at low temperatures, we have not extended the measurements at low NAD concentrations to low temperatures.

pH Dependence of the Isocitrate Dehydrogenase Reaction. Cennamo *et al.* (1970) studied at pH 6.3 the kinetics of yeast isocitrate dehydrogenase with NAD as the variable substrate. They suggest that the mechanism is random in the absence of 5′-AMP and ordered in its presence. Any conclusions about the mechanism at pH 7.5 might be unwarranted, however, because the critical concentration range, where one might observe cooperative ligand interactions, was not studied rigorously (*cf.* Atkinson, 1966).

At comparable substrate concentrations at pH 6.5 we found perfect agreement with the data of Hathaway and Atkinson (1963). A cooperative 5′-AMP effect is no longer observed at 0.15 mM isocitrate. The isotope effect at pH 6.5 as compared to pH 7.5 (Table III) indicated increased product discrimination and therefore faster steps behind the hydrogen transfer. This agrees with results obtained with phosphogluconate dehydrogenase, another enzyme catalyzing oxidative decarboxylation (Palm *et al.*, 1968b). At acid pH the release of one or both of the acid products (CO_2 and α-ketoglutarate) is probably favored. Since the size of the isotope effect is larger at pH 6.5 as compared to 7.5, it appears that these conditions indicate more clearly the maximal discrimination caused by a rate-limiting dehydrogenation step.

The closely similar isotope effects in the absence or presence of 5′-AMP at pH 6.5 (Table III) are compatible in both cases with a rate-limiting hydrogen transfer. An ordered reaction mechanism in the presence of 5′-AMP is excluded. We do not exclude, however, differences in the mechanism at isocitrate and NAD concentrations below half-saturation, as were seen at higher pH in the absence of 5′-AMP.

Comparison to Other Enzymes. Isotope effect studies with *threo*-D_s-[2-^{3}H]isocitrate in the reaction of NADP-specific isocitrate dehydrogenase from pig heart or ox heart were performed at pH 7.5 and 25°. Kinetic isotope effects of 1.40 and 1.32 were observed. No dependence on substrate concentrations was observed. It appears that the mechanisms of isocitrate dehydrogenases using a different coenzyme differ considerably (Londesborough and Dalziel, 1970) and can be differentiated by isotope effect studies (Katzendobler, 1971).

General Discussion

Earlier kinetic studies of NAD-dependent isocitrate dehydrogenase from yeast provided a model for strong cooperative interactions of substrates and modifiers leading to fourth order with respect to isocitrate and second order with respect to NAD, Mg^{2+}, and 5′-AMP. Rapid equilibration between

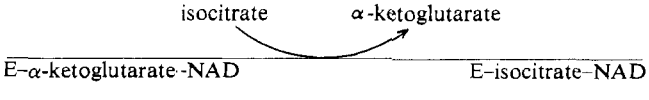
enzyme and substrate and a single rate-determining step were assumed. These assumptions agreed satisfactorily with experimental observations obtained at various ligand concentrations (Atkinson *et al.*, 1965). It was recently demonstrated that the kinetic order of the reaction with respect to isocitrate, AMP and metal ions is dependent on purity and modifications of the enzyme during purification (Kuehn *et al.*, 1970; Barnes *et al.*, 1971). We found, in addition to the ligand affinity interactions, an influence of substrates and modifiers on the kinetic mechanism and the temperature-dependent stability of enzyme conformations or enzyme–ligand complexes. These conclusions are based on the observation of different Arrhenius energies and the interpretation of these different states as a function of the size of the kinetic isotope effects.

At low NAD concentrations (0.16 mM) and varying isocitrate concentrations the activation energy calculated above 21° was approximately 9200 cal/mole. The high primary isotope effect of *threo*-D_s-[2-^{3}H]isocitrate under these conditions supports a random-order rapid-equilibrium mechanism, similar to yeast alcohol dehydrogenase (Mahler and Douglas, 1957; Palm, 1966) and as proposed by Atkinson *et al.* (1965). The notion that a single rate-limiting step obtains is further substantiated by the normal temperature dependence of the isotope effect above 21°.

At increasing isocitrate concentrations the isotope effect decreases slightly, from which we conclude that intermediate reaction steps behind the hydrogen transfer were slowed down. This will be discussed later.

At high NAD concentrations (1 mM) and varying isocitrate concentrations, the activation energy markedly increased compared to low NAD concentration. The observed value of 13,800 cal/mole is closely similar to liver alcohol dehydrogenase, which follows an ordered-reaction mechanism with rate-limiting coenzyme dissociation (Dalziel, 1963). A change of the rate-determining step(s) was also indicated by the substantial decrease of the kinetic isotope effect (Table III). The decrease was quantitatively explained by a model predicting slower rates of product dissociation compared to the rate of the hydrogen-transfer step. On the basis of the known affinity interactions of the substrates (and modifiers), it might be assumed that increasing substrate concentrations also lead to cooperative increase in product affinity, which would explain the slower product dissociation. The slow dissociation of one product will lead to an ordered product release, which might be adopted as the mechanism prevailing under these conditions.

This model has to be extended to explain the less pronounced decrease of the isotope effect in the presence of high NAD concentrations *and* high isocitrate concentrations (1 mM) as compared to low isocitrate concentrations. It was suggested that excess coenzyme could lead to alternative enzyme complexes of the type enzyme–oxidized product–oxidized coenzyme. In the case of liver alcohol dehydrogenase these complexes might either enhance or decrease product dissociation rates (Dalziel and Dickinson, 1966). In the case of isocitrate dehydrogenase, a reactivation of similar alternate product complexes by a second order reaction with isocitrate is readily envisualized



This sequence enhances the rate of product release in the presence of isocitrate, leading to a less pronounced decrease of the isotope effect.

In the presence of 5′-AMP, the kinetic isotope effect data and the temperature dependence of v_i are rather similar to the data in the presence of high NAD concentrations. It is possible that part of the 5′-AMP effect on the decreased isotope effect is mediated through the increased substrate affinity (K effect) because further changes on the isotope effect by an increase of isocitrate and NAD were not observed.

At pH 6.5 the isotope effect is somewhat larger than at pH 7.5 and influences of 5′-AMP are no longer observable at the substrate concentrations used (*cf.* Hathaway and Atkinson, 1963). We assume a faster release of products at low pH, supporting a random-order rapid-equilibrium mechanism with rate-limiting hydrogen transfer at pH 6.5.

Temperature-Dependent Changes and Stability of Enzyme Conformations. Recent studies of Barnes *et al.* (1971) showed that NAD^+-specific isocitrate dehydrogenase from yeast can be dissociated into subunits of a molecular weight of 39,000/subunit. At 20°, the enzyme probably contains eight subunits. Our present estimate of the molecular weight (236,000) by Sephadex G-200 chromatography, obtained from a less purified preparation, is considerably lower. It is conceivable, however, that both estimates of the molecular weight of the oligomer represent different aggregation states, *e.g.*, eight and six subunits, respectively. Our evidence for a subunit structure of the yeast enzyme came from quite distinct observations. Among other regulatory enzymes with a known subunit structure, dimeric skeletal muscle phosphorylase undergoes a transition to a different conformation around 13°. This cold inactivated enzyme is also characterized by a higher activation energy of the enzyme reaction and reversibility of the transition; 5′-AMP stabilizes the high temperature conformation (Graves *et al.*, 1965; Kastenschmidt *et al.*, 1968). These properties are similar to those observed with isocitrate dehydrogenase, only we found a transition temperature of 21° at low NAD concentration and ~9.5° at high NAD concentration or in the presence of 0.2 mM 5′-AMP. Temperature-dependent conformational transitions can also be observed, however, if no quarternary structure changes occur (Massey *et al.*, 1966). Therefore we cannot define at this point if quarternary structure changes contribute to the observed differently active forms of isocitrate dehydrogenase.

Our present observations about conformational states and their temperature stability allow the following conclusions. In absence of 5′-AMP two catalytically distinct conformations or enzyme–substrate complexes at either "low NAD" or "high NAD" can be distinguished. Both are converted to catalytically less active conformational states on lowering temperature to 21 and 9.5°, respectively. On binding of 5′-AMP to isocitrate dehydrogenase the functional properties seem to correspond closely to the "high NAD" conformation; however, the property of permitting alternate kinetic pathways by interaction with high NAD concentrations seems to be lost. The protective function of 5′-AMP against a cold sensitive conformational change shows the important contribution of 5′-AMP as a conformational determinant.

The low-temperature conformations exhibit in all cases a markedly increased activation energy, which is not due to an irreversible denaturation (desensitization) of the protein. Because the kinetic isotope effects allow one to conclude that a rate-limiting hydrogen transfer is obligatory for distinct low- and high-temperature conformations, the transition-state coordinates of the rate-limiting complex must be quite different in these cases. This offers a promising way to study the

altered participation of the same protein in the active complex. As pointed out earlier, the decrease of isotope effects in the reaction of the same substrate can be explained by a more product-like arrangement of the transition state coordinates.

A further indication of the possible participation of different protein conformations in the catalytic process is given by a comparison with NADP-specific dehydrogenases. These enzymes follow a compulsory-order mechanism (Londesborough and Dalziel, 1970) with substrate K_M's similar to AMP-activated NAD-specific isocitrate dehydrogenase. This mechanism requires small kinetic isotope effects as were found in our present studies. It appears that during evolution two different routes were pursued to select an enzyme with a compulsory-order-type mechanism. In case of the yeast NAD-specific enzyme 5′-AMP stabilizes this conformation.

Compared to the "classical" models for allosteric enzymes, the kinetics of yeast isocitrate dehydrogenase do not fit the predictions for a K system: differential affinities of substrate and modifiers toward different enzyme states without intrinsic rate changes (Monod *et al.*, 1965). As could be shown in the present study, the altered affinity by ligand interactions is concomitant with changes in the reactivity of the ligand, probably due to differential changes in the structural arrangement at the ligand binding site. This raises some doubt as to whether K systems of the former, rather restricted classification actually exist, since conformational changes, symmetric or sequential which alter affinity will also alter kinetic parameters. One exception, however, is readily comprehended. If either the (preexisting) T or R state has no catalytic activity, then only one mechanism can be devised and the velocity is solely dependent from the fractional saturation by substrates. Hopefully, sensitive methods, such as fast-reaction kinetics or kinetic isotope effects will demonstrate more examples of changes in the reaction mechanism in allosteric "K systems."

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