Oxygen and Deuterium Exchanges Show Reversal of Catalytic Steps of Citrate Synthase: Catalytic Cooperativity Is Not Observed†

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ABSTRACT: The oxygen and deuterium exchange reactions of citrate synthase have been examined. During steady-state citrate formation from ^{18}O carbonyl oxygen or [^{2}H]methyl-labeled acetyl-CoA, some exchange with water oxygen and hydrogen occurs. The product citrate acquires 1.26 water oxygens (1.00 is required by the reaction) and 0.35 water hydrogen (none are required by the reaction). From statistical considerations, both these values represent about 0.7 reversal prior to product release at their respective exchange steps. The extent of the exchanges is not altered over a wide range of acetyl-CoA or oxalacetate concentrations or at ATP concentrations that inhibit catalysis by 75%. The results show that during net catalysis by citrate synthase a small amount of reaction reversal occurs at both the condensation and hydrolysis steps. No catalytic cooperativity between subunits of this dimeric enzyme is evident. Some mechanistic implications of these results are discussed.

Citrate synthase, first described by Stern & Ochoa (1951), catalyzes the initial reaction in the citric acid cycle. The pig heart enzyme, with a molecular weight of about 100 000, consists of two identical subunits that are inactive upon dissociation (Wu & Yang, 1970). The equilibrium strongly favors citrate formation (Stern et al., 1952). The enzyme catalyzes the condensation of acetyl-CoA and oxalacetate to form an intermediate, citryl-CoA (Eggerer & Remberger, 1963; Srere, 1963), which is subsequently hydrolyzed to form the product citrate.

These two separate enzymic activities, condensation and hydrolysis, make citrate synthase a good candidate for isotope exchange studies since there are two partial reactions where exchange could occur by reversal of the reaction steps. The first step in the reaction sequence is considered to involve enolization of acetyl-CoA, which does not occur with enzyme alone but occurs in the presence of oxalacetate (Marcus & Vennesland, 1958; Bové et al., 1959; Srere, 1967). When the reverse reaction occurs in the presence of $^{3}H_2O$, tritium incorporation into the methyl group of acetyl-CoA is observed (Bové et al., 1959). An exchange reaction is observed at this step when (*S*)-malate, which cannot condense with acetyl-CoA, is substituted for oxalacetate in the presence of $^{3}H_2O$ (Eggerer, 1965). A small isotope effect is observed with [^{2}H]acetyl-CoA (Kosicki & Srere, 1961a).

The hydrolysis of the enzyme-bound citryl-CoA must give incorporation of at least one water oxygen into the C-5 carboxyl group, a result in accord with the observed incorporation of ^{18}O from H_2O into citrate (Suelter & Arrington, 1967; Wunderwald & Eggerer, 1969). Reversal of the hydrolysis prior to citrate release would give oxygen exchange.

Probability considerations have been used to estimate the number of reaction reversals at catalytic sites responsible for exchange of oxygens into phosphate or carboxylate oxygens of products from ATP-linked synthetase reactions (Hackney & Boyer, 1978; Bild & Boyer, 1980). In these studies with synthetases it was observed that the lowering of substrate concentrations increases the extent of oxygen exchange into each product molecule released. These data are indicative of subunit interactions, where the binding of the substrate at one catalytic site promotes catalytic events at an alternate site (catalytic cooperativity). The same type of treatment was applied here to the exchange data of citrate synthase.

Experimental Procedures

Pig heart citrate synthase was obtained from Sigma Chemical Co., St. Louis, MO. Where indicated, it was passed through a centrifuge–Sephadex column (Penefsky, 1977) equilibrated with an appropriate reaction mixture. [^{18}O]Citrate was prepared by equilibration of citric acid with highly labeled $H^{18}OH$ at pH 1.0 and 110 °C for 48 h. For preparation of ^{18}O- and ^{2}H-labeled acetyl-CoA, the required acetic anhydride was prepared by heating labeled sodium acetate with phenylsulfonyl chloride (Bloch & Rittenberg, 1947) in a sealed glass tube. The anhydride that was formed was transferred under reduced pressure to a frozen solution of CoASH in 1 M $NaHCO_3$, followed by thawing to allow acetylation of the CoASH. Completion of acetylation was confirmed by measurement of SH groups, and the acetyl-CoA concentration was determined enzymically.

The exchange reactions were run under conditions of net citrate synthesis at 270 °C and pH 8.2 in a final volume of 1.0 mL with reaction mixtures as given in the table legends. The substrate was either included in the reaction mixture or added at a constant, known rate to stirred samples through a Sage Instruments syringe pump, Model 341A, fitted with a 25-μL Hamilton syringe. With samples in which the pump was not used, the progress of the reaction was monitored by observing the reduction of DTNB[1] by the product CoASH spectrophotometrically at 412 nm (Srere et al., 1963). After production of 50–100 nmol of citrate the reaction was quenched by mixing the solution with an equal volume of either cold $CHCl_3$ or cold 40 mM NaOH. The citrate and oxalacetate were adsorbed on a 2 × 0.5 cm Dowex AG 1-X4 anion-exchange column. The column was washed with 10 mM HCl, and the adsorbed acids were eluted with 50 mM HCl. The first milliliter of effluent was discarded, and the next 1.5 mL, which contained the citric acid, was collected into a tube containing 25 μL of concentrated NH_4OH. The conversion

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[1] Abbreviations: DTNB, 5,5′-dithiobis(2-nitrobenzoic acid); Tris, tris(hydroxymethyl)aminomethane.

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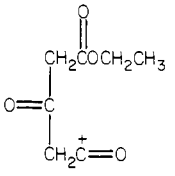
to the ammonium salt was necessary to avoid some exchange of carboxylate oxygens. The sample was lyophilized, and then the ammonium citrate was reconverted to the acid in a non-aqueous environment, to again minimize the exchange of carboxylate oxygens. This was accomplished by mixing with dried solid $KHSO_4$ and absolute ethanol. Using the method described here, control experiments with [^{18}O]citrate showed an overall loss of label of no more than 4%.

The supernatant ethanolic solution was transferred to a Pierce "Reacti-Vial" and the solvent evaporated. The acid was converted into the volatile triethyl derivative by treatment with diazoethane in ether. Unreacted diazoethane and the ether were blown off with N_2, and the derivative was taken up in 50 μL of CH_2Cl_2. Approximately 1-μL samples were injected into a Hewlett-Packard 5995A GC–mass spectrometer. The gas chromatograph, which separated ethylated citric acid from the ethylated oxalacetic acid, was operated isothermally at 204 °C with a 6 ft × 2 mm glass column packed with a 3% OV-275 on a 40–60-mesh Chromosorb T support and eluted with helium at a flow rate of 30 mL/min. Fragmentation of the ethyl citrate gave a base peak at m/e 157 with a retention time of 2.00 min that was used for measurement of ^{18}O content.

[^{18}O]- and [^{2}H]Citrate Analysis. For analysis of the derivatized [^{18}O]citrate it was necessary to know how many oxygens from the C-1 and C-5 carboxyl groups of citrate were present in the m/e 157 fragment. From the mass distribution obtained with an [^{18}O]citrate standard labeled in all carboxyl oxygens, it was clear that this fragment contained three such carboxyl oxygens. An assignment of structure I was made based on likely rearrangements and corroborated by high-resolution mass spectrometry (data not shown).



I

The average number of water oxygens appearing in citrate, $\bar{O}'$, was calculated from eq 1, where ^cF_o and ^aF_o are the ob-

$$\bar{O}' = 2 - (4/3)^cF_o/^aF_o \quad (1)$$

served oxygen atom fractions of the ethyl citrate m/e 157 fragment and the molecular ion of the acetate group of acetyl-CoA, respectively. The factor of 4/3 arises from the asymmetry of the m/e 157 fragment. The average number of water hydrogens appearing in product citrate per citrate released, $\bar{H}'$, was calculated from eq 2, where ^aF_d and ^cF_d are

$$\bar{H}' = 1.1[2 - 2(^cF_d)/^aF_d] \quad (2)$$

the observed deuterium atom fractions of the m/e 157 fragment of ethyl citrate and the molecular ion of the acetyl group of acetyl-CoA, respectively. The factor of 1.1 corrects for the observed isotope effect of 1.3 (see Results); it is the ratio of the probabilities of the loss of deuterium with an isotope effect (1/3.3) and without an isotope effect (1/3). Since the final number of reversals is small (see Results), the observed isotope effect of 1.3 is valid in this calculation. The distributions were corrected for natural abundance ^{13}C spillover prior to calculating the deuterium atom fraction.

Estimation of the Number of Reversals during Exchange. The calculations outlined below are made assuming that exchange is a reflection of reaction reversal at the catalytic site (see Discussion). For explanation of the approach used, Figure 1 is useful. In calculations involving deuterium, the methyl

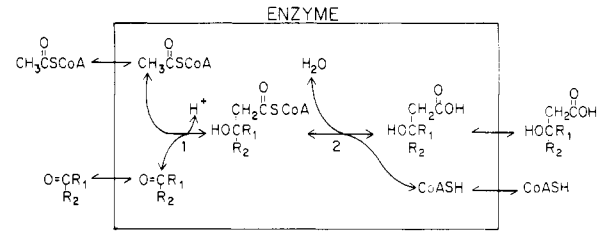


FIGURE 1: Citrate synthase reaction. One subunit of the dimer is depicted. R_1 is COOH and R_2 is CH_2COOH. Steps 1 and 2 are potential places for exchange.

group of acetyl-CoA is considered initially to be uniformly labeled with deuterium. Net citrate synthesis does not require incorporation of water hydrogen into the intermediate citryl-CoA or into the product citrate. The three methyl deuteriums of enzyme-bound acetyl-CoA are considered to be torsiosymmetric. If the reaction reverses, the departing oxalacetate is replaced by a protium and the enzyme-bound acetyl-CoA would contain two deuteriums and one protium. Protium removal would result in a deuterium atom fraction of the product citrate identical with that of the original [^{2}H]acetyl-CoA. Deuterium removal would result in a decrease of the deuterium atom fraction of the product citrate. After an infinite number of reversals no deuterium would remain in the product citrate. This calculation assumes no isotope effect; as mentioned above, the determination of the observed value of $\bar{H}'$ is corrected for the isotope effect.

The predicted value of $\bar{H}'$ can be represented by eq 3 (Kohlbrenner, 1980):

$$\bar{H}' = \sum_{n=0}^{\infty} H_n(1 - P_c)P_c^n \quad (3)$$

H_n is the average number of water hydrogens incorporated into citric acid after n reversals and P_c is the probability of reversal of step 1 in Figure 1. This value is identical with the partition coefficient described elsewhere (Boyer & Hackney, 1978). The value $(1 - P_c)P_c^n$ represents the probability that n reversals will occur before product release. H_n can be represented by eq 4

$$H_n = ab + cd \quad (4)$$

where a and c are the probabilities that a deuterium and hydrogen, respectively, will be released to the medium and b and d are the number of hydrogens in citrate after reversal n if the nth reversal involves a deuterium and hydrogen, respectively. Using probability considerations, this can be expressed in terms of H_n (eq 5). Equation 5 reduces to eq 6.

$$H_n = [(2 - H_{n-1})/3](H_{n-1} + 1) + [(1 + H_{n-1})/3]H_{n-1} \quad (5)$$

$$H_n = (2/3)(1 + H_{n-1}) \quad (6)$$

Since the initial value of H_n is known, eq 7–9, etc., can be used

$$H_0 = 0 \quad (7)$$

$$H_1 = 2/3 \quad (8)$$

$$H_2 = 2/3 + (2/3)^2 \quad (9)$$

$$\vdots$$

$$H_n = \sum_{i=0}^{n}(2/3)^i - 1 \quad (10)$$

$$H_n = 3[1 - (2/3)^{n+1}] - 1 \quad (11)$$

$$\bar{H}' = 3/(3 - 2P_c) - 1 \quad (12)$$

to generate the expression given in eq 10, which can be rewritten as eq 11. By substitution into eq 3, a value of $\bar{H}'$ can be obtained (eq 12). This equation shows the requisite limits;

the value of $\bar{H}'$ is equal to 0 in the absence of exchange ($P_c = 0$) and is equal to 2 in the presence of infinite exchange ($P_c = 1$). The number of reversals at the deuterium exchange step, R_d, can be estimated (Hackney & Boyer, 1978) by substituting the value of P_c [eq 13 (Hackney & Boyer, 1978)] into eq 12 and solving for R_d in terms of $\bar{H}'$ (eq 14).

$$P_c = R_d/(1 + R_d) \quad (13)$$

$$R_d = 3\bar{H}'/(2 - \bar{H}') \quad (14)$$

A similar method can be applied to $\bar{O}'$, the average number of water oxygens that appear in citrate per citrate released, yielding eq 15.

$$R_o = (2\bar{O}' - 2)/(2 - \bar{O}') \quad (15)$$

Predicted Distributions of [^2H]Citrate Species. Citrate formed from [^{2}H]acetyl-CoA could contain a maximum of two deuteriums. On the basis of this value and probability considerations, the predicted distribution can be calculated for a given $\bar{H}'$ value. The relative amount of ^2H_n is given by eq 16, where n is equal to 0, 1, or 2. The ^aF_d term is multiplied

$$^2H_n = \left[\frac{2!}{n!(2-n)!}\right]\left(^aF_d\frac{2-\bar{H}'}{2}\right)^n\left(1 - {}^aF_d\frac{2-\bar{H}'}{2}\right)^{2-n} \quad (16)$$

by $(2 - \bar{H}')/2$ to give the effective atom fraction during the reaction. After calculation of the distribution from eq 16, corrections are made for ^{13}C natural abundance. The sum of these values is equal to 1. Since this method is based solely on probability, large deviations from the predicted distribution would indicate the presence of more than one reaction pathway.

Results

Experimental Design. A schematic representation of a possible sequence for the citrate synthase reaction is depicted in Figure 1, showing the substrate binding, interconversion, and product release steps. Steps 1 and 2 refer to two separate potential exchange reactions for this sequence.[2] During the net reverse reaction (acetyl-CoA synthesis), the reverse of the condensation reaction (step 1) would result in one water proton appearing in the methyl group of acetyl-CoA. During net citrate synthesis, hydrolysis of the intermediate citryl-CoA (step 2) would result in one water oxygen appearing in the C-5 carboxyl group of citrate. If reversal of step 1 during net citrate synthesis occurs prior to citrate release, water protons would be incorporated into the C-4 methylene group of citrate. If reversal of step 2 occurs prior to citrate release, more than the one water oxygen required for the reaction would be incorporated into the C-5 carboxyl group of citrate. The number of reaction reversals giving exchange could range from zero to infinity; this would yield values for the number of water protons appearing in citrate from reversal of step 1 from zero to two and the number of water oxygens appearing in citrate from reversal of step 2 from one to two. Thus, exchange at these individual steps can be monitored by using labeled substrates.

In order for exchange to occur, it is necessary to have freedom of rotation in such a manner that all methyl protons of enzyme-bound acetyl-CoA (step 1) or both C-5 carboxyl oxygens of enzyme-bound citric acid (step 2) can participate in the reaction. Exchange of the hydrogens of the methyl groups has been demonstrated by using a substrate analogue (Eggerer, 1965). The γ-carboxyl group of *Escherichia coli* glutamine synthetase bound glutamate was shown to be free to rotate (Stokes & Boyer, 1976); the carboxyl group of enzyme-bound citrate may behave similarly.

[2] As noted under Discussion, other possibilities for the proton exchange also need consideration.

Table I: Extent of Oxygen Exchange at Varying Substrate Concentrations during Citrate Formation Catalyzed by Citrate Synthase[a]

[^{18}O]acetyl-CoA (μM)	oxalacetate (μM)	enzyme[b] (unit/mL)	$\bar{O}'$[d]	R_o[e]
430	3000	0.08	1.27	0.74
30	3000	0.08	1.25	0.67
8	3000	0.08	1.30	0.86
2	3000	0.08	1.33	0.98
1	3000	0.08	1.27	0.74
<0.2[f]	3000	0.39[c]	1.26	0.70
<0.05[f]	3000	1.56[c]	1.26	0.70
120	20	0.01[c]	1.25	0.67
120	<0.2[g]	0.06[c]	1.25	0.67
120	<0.05[g]	0.26[c]	1.26	0.70

[a] Reaction mixtures contained 100 mM Tris–acetate (pH 8.2), 250 μM DTNB, and the indicated concentration of [^{18}O]acetyl-CoA (0.628 atom fraction), oxalacetate, and citrate synthase in a final volume of 1.0 mL. Reactions were stopped by the chloroform method. [b] One unit is defined as the amount of enzyme catalyzing 1 μmol/min at 27 °C under optimal conditions (the protein concentration ranged from 0.1 to 14 μg/mL). [c] Citrate synthase was equilibrated with buffer by use of a centrifuge–Sephadex column (Penefsky, 1977). [d] $\bar{O}'$ is the average number of water oxygens appearing in citrate per citrate released. [e] R_o is the estimated average number of reaction reversals of the oxygen incorporation step that had occurred before product dissociation. [f] These values were estimated from calibration with 0.08 unit of enzyme/mL and the expected decrease in the steady-state level of substrate with the increase in enzyme concentration. [g] As in footnote *f*, but estimated from calibration with 0.01 unit of enzyme/mL.

Regulation of the Steady-State Substrate Concentration. In exchange experiments at substrate concentrations below 20 μM the substrate was delivered at a constant rate through a syringe pump to a stirred reaction mixture in a system similar to one previously described (Bild & Boyer, 1980). For determination of the flow rate necessary to maintain a given substrate concentration, 10 mM [^{18}O]acetyl-CoA, [^{2}H]-acetyl-CoA, or oxalacetate was delivered to a complete reaction mixture at various preset rate positions. After the attainment of steady state, the pump was shut off, the sample placed in the spectrophotometer, and the amount of continued reaction determined by monitoring the reduction of DTNB by product CoASH. The concentration of substrate was extrapolated to the time at which the pump was turned off to determine the effective substrate concentration during steady state. In some samples the effective substrate concentration was further decreased by increasing the enzyme concentration.

^{18}O Exchange during Net Citrate Synthesis. The extent of oxygen exchange during net synthesis was measured by using [^{18}O]acetyl-CoA at varying substrate concentrations, as shown in Table I. The median of the average number of water oxygens present in each citrate molecule produced, $\bar{O}'$, was 1.26 for all substrate concentrations. This value shows that definite reversal of the oxygen exchange step occurs. An approach similar to that of Kohlbrenner (1980) based on probability considerations was used to estimate the extent of reversal of the hydrolytic step (see Experimental Procedures). The median of R_o, the average number of reversals of the oxygen exchange step prior to product release, was 0.7.

The oxygen exchange accompanying net citrate formation is dependent on conditions allowing net catalysis. Exchange

Table II: Percent Distribution and Extent of Deuterium Exchange during Citrate Formation Catalyzed by Citrate Synthase[a]

observed % distribution[b]			theoretical % distribution[c]				
$^2H_0$	$^2H_1$	$^2H_2$	$^2H_0$	$^2H_1$	$^2H_2$	$\bar{H}'$	R_d
5.1	30.3	64.6	4.1	32.3	63.6	0.38	0.70

[a] The reaction mixture contained 100 μM [^{2}H]acetyl-CoA (0.969 atom fraction), 100 μM oxalacetate, 250 μM DTNB, and 20 mM Tris-acetate (pH 8.2). The reaction was initiated with 0.04 unit of centrifuge-Sephadex column-treated citrate synthase, giving a final volume of 1.0 mL. The reaction was quenched by using the NaOH method. [b] Relative distribution of the deuterated species. $^2H_0$ refers to citrate containing zero deuteriums, $^2H_1$, one deuterium, etc. This distribution was corrected for spillover of natural abundance ^{13}C. [c] Predicted distribution for the observed deuterium atom fraction.

was not observed when citrate was incubated with the enzyme even with added CoASH. This gives evidence that the exchange does not result from binding of citrate once released. Also, possible occurrence of exchange by reversal of the overall reaction with CoASH is unlikely because the thiol was removed as soon as it was formed by reaction with DTNB.

As can be seen from Table I, wide concentration ranges above and below K_m values of either [^{18}O]acetyl-CoA or oxalacetate did not significantly change the number of reversals. The literature value of the K_m for acetyl-CoA is approximately 20 μM (Kosicki & Srere, 1961b), and the literature value of the K_m for oxalacetate ranges from 1 to 16 μM (Spector, 1972).

Deuterium Exchange during Net Citrate Synthesis. The extent of deuterium exchange was measured during net citrate synthesis using [^{2}H]acetyl-CoA. An isotope effect of 1.3 was observed (data not shown). Table II shows the results of a typical deuterium exchange experiment. The median of the average number of water hydrogens present in citrate for each citrate produced, $\bar{H}'$, was 0.35. The average number of reversals at the deuterium exchange step, R_d, was thus about 0.7 (see Experimental Procedures).

Also shown in Table II is the percent distribution of the various deuterated species of citrate of this sample experiment. This distribution has been corrected for ^{13}C natural abundance spillover. Unlike the oxygen exchange in which only two species are observed, the deuterium exchange results in three species. Thus, the distribution can be compared to a predicted distribution that assumes equivalence of exchangeable deuteriums and is based strictly on probability. If exchange is a result of two pathways with different relative rates of exchange and product release, a different distribution would be observed (Hutton & Boyer, 1979). The predicted distribution shown in Table II (see Experimental Procedures) is close to the observed distribution.

The deuterium exchange was also measured at varying substrate concentrations, shown in Table III. The distributions, not shown here, were close to the predicted distributions for single reaction pathways. Over a 2000-fold concentration range no differences in extent of proton exchange per molecule of citrate released were observed for either substrate.

Deuterium Exchange during Partially Inhibited Citrate Synthesis. Citrate synthase is readily inhibited by ATP. As this inhibition is competitive with acetyl-CoA it seems likely that ATP binds at the catalytic site. The possibility exists, however, that the competitive interaction could result from ATP binding at a control site and that resultant conformational change could modify rate constants and substrate dependency

Table III: Extent of Deuterium Exchange at Varying Substrate Concentrations during Citrate Formation Catalyzed by Citrate Synthase[a]

[^{2}H]acetyl-CoA (μM)	oxalacetate (μM)	enzyme (unit/mL)	$\bar{H}'$	R_d
100	100	0.04	0.38	0.70
8	100	0.04	0.40	0.73
<0.2	100	0.40	0.38	0.70
<0.05	100	0.53	0.40	0.73
60	100	0.01	0.38	0.70
100	8	0.01	0.40	0.75
100	<0.2	0.06	0.36	0.66
100	<0.05	0.24	0.37	0.68

[a] The reaction mixtures contained 20 mM Tris-acetate (pH 8.2), 250 μM DTNB, and the indicated amounts of 0.969 atom fraction [^{2}H]acetyl-CoA, oxalacetate, and centrifuge-Sephadex column-treated citrate synthase in a final volume of 1.0 mL. The reactions were quenched by using the NaOH method. For other details, see the legend to Table I.

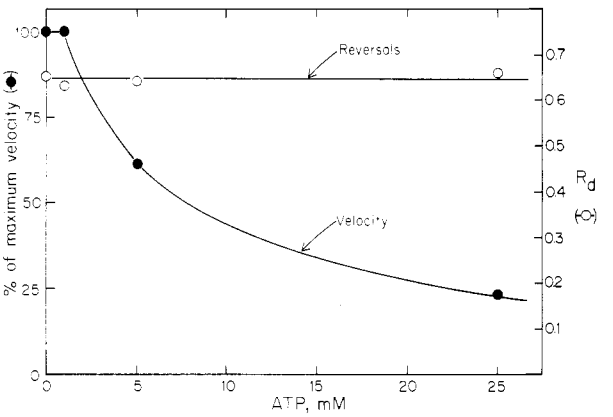


FIGURE 2: Extent of reversal during citrate synthesis in the presence of ATP. The reaction mixture was as described in the legend in Table III and contained 60 μM [^{2}H]acetyl-CoA, 100 μM oxalacetate, and the indicated amounts of ATP.

of the deuterium and oxygen exchanges. However, at ATP concentrations that inhibit the enzyme up to 75%, no change in the number of reversals is observed (Figure 2). This was also true at [^{2}H]acetyl-CoA and oxalacetate concentrations varied as in Table III (data not shown).

Discussion

The experimental results show that during net citrate synthesis catalyzed by citrate synthase, appreciable amounts of deuterium and oxygen exchange occur (Tables I and II). The extent of exchange is similar for both isotopes and is not affected by substrate concentrations over a 2000-fold concentration range (Tables I and III). It is also not affected by ATP at inhibitory concentrations (Figure 2).

Three possible explanations for the deuterium exchange need consideration. One is that exchange is induced at the catalytic site prior to citryl-CoA formation. Although acetyl-CoA in the presence of the enzyme does not show exchange of the methyl hydrogens with water (Marcus & Vennesland, 1958), the enzyme is regarded as inducing enolization to favor the condensation reaction (Srere, 1967; Eggerer et al., 1970; Rêtey et al., 1970). This would be somewhat analogous to the formation of an enzyme-bound carbanion prior to product formation as has been implicated in enolase catalysis (Dinovo & Boyer, 1971).

A second possibility is that exchange results from reversal of the reaction forming citryl-CoA. This is as indicated in Figure 1. During reversal one proton from water must appear

in the methyl group, and because of expected torsional symmetry of the methyl group, the next bound citryl-CoA formed could contain a hydrogen derived from water.

A third, less likely possibility is that proton exchange might accompany the cleavage of citryl-CoA. The cleavage could be visualized as occurring by elimination of the CoASH, followed by hydration of the substituted ketene formed. Protonation of the α-carbon could be partially from a basic group on the enzyme that is protonated during the elimination and partially shielded from solvent, giving rise to incorporation of less than one solvent proton per citrate released. If the same conditions that favored incorporation of a solvent proton also favored reversal of the OH^- addition to the carbonyl, the approximately equivalent number of reversals of the two exchange steps could be explained.

Irrespective of the exchange mechanism, the distribution of deuterium remaining in the citrate (cf. Table II) points to involvement of only one catalytic pathway. This means that each acetyl-CoA molecule that binds faces equal probability of exchange; the exchange observed does not result from part of the substrate reacting in a pathway with compulsory exchange and part in a pathway with no exchange.

For any of the proton exchange mechanisms the number of reversals we estimate is a minimum for the actual number that may occur. The carboxyl group must show some rotation or torsional symmetry, but nonetheless hindered rotation could limit exchange. Also, the proton departing from substrate could be transferred to an enzyme basic group partially inaccessible to solvent and then returned to the reaction intermediate. For example, this type of shielded proton has been proposed to explain the exchange properties of glyoxalase (Hall et al., 1976).

The oxygen exchange likely results from reversal of the hydrolysis of citryl-CoA as depicted in Figure 1 (step 2). Exchange at the catalytic site requires equivalence of the carboxyl oxygens. The carboxyl oxygens of succinate in the succinyl-CoA synthetase reaction showed increased exchange at low substrate ATP concentrations (Bild et al., 1980); the carboxyl oxygens of glutamine synthetase bound glutamate were shown to be equivalent (Stokes & Boyer, 1976), and increased exchange was observed at low substrate NH_3 concentration (Bild & Boyer, 1980). But it is also possible that the oxygen exchange occurs with enzyme-bound citrate after its formation and prior to its release; citrate carboxyl groups show relatively facile exchange as contrasted to acetate (Dole, 1952; this paper). However, this is unlikely in light of the lack of exchange observed when citrate is incubated with the enzyme with or without added CoASH; a previously undetected enzyme isomerization step would be necessary to support this possibility.

Our value of $\bar{O}'$, the number of water oxygens that appear in the product citrate during synthesis, is 1.26. Earlier workers reported that about 1.0 oxygen is incorporated (Suelter & Arrington, 1967; Wunderwald & Eggerer, 1969). They ran their reactions in low abundance $H_2{}^{18}O$, decarboxylated the product, and analyzed the labeled CO_2. Our present procedures, which employ substrates containing high atom fractions of isotopes and direct mass spectrometry of isolated ethyl citrate, may be more accurate. Perhaps more important, extensive corrections were made by both groups to account for the facile nonenzymic exchange that occurs under acidic conditions (Dole, 1952). Our method of isolation eliminated the need for such corrections. In earlier work a principal objective was to find whether or not water oxygens were incorporated. Later developments have led to the recognition of fractional incorporations and the importance of more precise evaluations.

The constant value of the extent of exchange at varying substrate concentrations in citrate synthase is in sharp contrast to the behavior of the mitochondrial ATP synthase (Hackney & Boyer, 1978) and ATPase (Hutton & Boyer, 1979), the chloroplast ATP synthase (Hackney et al., 1979), and ATPase (Kohlbrenner, 1983), succinyl-CoA synthetase (Bild et al., 1980), and glutamine synthetase (Bild & Boyer, 1980). All of these enzyme reactions show substrate modulation of an exchange reaction, which was interpreted as indicating alternating site activity in which binding at one catalytic site promotes product release from an alternate site. These enzymes also show a tendency for negative cooperativity of substrate binding or half-of-sites reactivity. The Ca^+-activated CF_1 shows no such modulation (Kohlbrenner & Boyer, 1983), nor does the yeast plasma membrane ATPase (Amory et al., 1982); the latter does not exhibit negative cooperativity of substrate binding (Dufour & Goffeau, 1980). Such is also the case for citrate synthase; acetyl-CoA has been proposed to decrease the velocity of oxalacetate binding (Kosicki & Srere, 1961b; Johansson & Pettersson, 1977) but no negative cooperativity of substrate binding is observed.

The evidence in this paper for facile reversal of catalytic steps with bound reactants may reflect a general property commonly expressed by enzyme catalytic sites: a tendency to maintain roughly equal concentrations of enzyme-bound intermediates and to promote their rapid interconversion. Such properties were evident as part of the experience in early isotope exchange studies with alcohol dehydrogenase (Silverstein et al., 1964) and served as the basis for the first suggestions of the facile oxygen exchanges and retention of catalytic site ATP by ATP synthase and myosin ATPase (Boyer et al., 1973). The emerging generalization has been emphasized by Albery & Knowles (1976).

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Inhibition of Pyruvate Dehydrogenase Multienzyme Complex from *Escherichia coli* with a Radiolabeled Bifunctional Arsenoxide: Evidence for an Essential Histidine Residue at the Active Site of Lipoamide Dehydrogenase†

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ABSTRACT: Incubation of pyruvate dehydrogenase multienzyme complex (PD complex) from *Escherichia coli* with thiamin pyrophosphate, pyruvate, coenzyme A, Mg^{2+}, and the radiolabeled bifunctional arsenoxide *p*-[(bromoacetyl)amino]phenyl arsenoxide ($BrCH_2{}^{14}CONHPhAsO$) led to the irreversible loss of lipoamide dehydrogenase (E3) activity. The mode of inactivation occurred by initial "anchoring" of the reagent via its –AsO group to reduced lipoyl residues on lipoate acetyltransferase (E2) (generated by substrates) followed by the delivery of the $BrCH_2{}^{14}CO$– moiety into the active site of E3 where an irreversible alkylation ensued [Stevenson, K. J., Hale, G., & Perham, R. N. (1978) *Biochemistry 17*, 2189]. To account for nonspecific alkylations, not mediated by this delivery process, control experiments were conducted in which the radiolabeled bifunctional reagent was incubated with PD complex in the absence of substrates. E3 subunits were isolated from inhibited and control PD complexes by chromatography on hydroxylapatite in the presence of 8 M urea. Acid hydrolysis of the alkylated E3 and control E3 samples produced radiolabeled carboxymethylated amino acids that were identified and quantitated by high-voltage electrophoresis and amino acid/radiochemical analysis. The inhibited sample contained N^3-(carboxymethyl)histidine and a small amount of *S*-(carboxymethyl)cysteine. These residues were not present in significant amounts in the controls. The loss of 81% of E3 activity correlated with the alkylation of about 0.7 residue of histidine and 0.1 residue of cysteine per mol of E3.

The pyruvate dehydrogenase multienzyme complex (PD complex)[1] from *Escherichia coli* catalyzes the overall reaction

$$\text{pyruvate} + NAD^+ + \text{CoASH} \rightarrow \text{acetyl-CoA} + NADH + H^+ + CO_2$$

The complex consists of three different enzymes, which in order of partipication are pyruvate dehydrogenase (lipoate) (E1) (EC 1.2.4.1), lipoate acetyltransferase (E2) (EC 2.3.1.12), and lipoamide dehydrogenase (NADH) (E3) (EC 1.6.4.3) (Reed, 1974; Hucho, 1975; Perham, 1975). A total of 24 apparently identical E2 chains form the structural core of the complex (Reed & Oliver, 1968; Reed, 1974). Evidence suggests that two lipoyl residues are present per polypeptide chain of E2 (Danson & Perham, 1976; Brown & Perham, 1976; Speckhard et al., 1977; Collins & Reed, 1977; Bates et al., 1977; White

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[1] Abbreviations: PD complex, pyruvate dehydrogenase multienzyme complex; TPP, thiamin pyrophosphate; HVE, high-voltage electrophoresis; $BrCH_2{}^{14}CONHPhAsO$, *p*-[(bromo[^{14}C]acetyl)amino]phenyl arsenoxide; 1-CM-His, N^1-(carboxymethyl)histidine; 3-CM-His, N^3-(carboxymethyl)histidine; 1,3-CM-His, N^1,N^3-bis(carboxymethyl)-histidine; N^ϵ-CM-Lys, N^ϵ-(carboxymethyl)lysine; S-CM-Cys, *S*-(carboxymethyl)cysteine; S-CM-Homocys, *S*-(carboxymethyl)homocysteine; $NaDodSO_4$, sodium dodecyl sulfate; BAL, 2,3-dithiopropanol (British Anti-Lewisite); NMR, nuclear magnetic resonance; EDTA, ethylenediaminetetraacetic acid.

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