Kinetic Characterization of Yeast Pyruvate Carboxylase Isozyme Pyc1 and the Pyc1 Mutant, C249A[†]

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ABSTRACT: The yeast Pyc1 isoform of pyruvate carboxylase has been further characterized and shown to differ from the Pyc2 isoform in its K_a for K^+ activation. Pyc1 differs from chicken liver pyruvate carboxylase in the lack of effect of acetyl-CoA on ADP phosphorylation by carbamoyl phosphate, which may be a result of differences in the loci of action of the effector between the two enzymes. Solvent D_2O isotope effects have been measured with Pyc1 on the full pyruvate carboxylation reaction, the ATPase reaction in the absence of pyruvate, and the carbamoyl phosphate-ADP phosphorylation reaction for the first time for pyruvate carboxylase. Proton inventories indicate that the measured isotope effects are due to a single proton transfer step in the reaction. The inverse isotope effects observed in all reactions suggest that the proton transfer step converts the enzyme from an inactive to an active form. Kinetic measurements on the C249A mutant enzyme suggest that C249 is involved in the binding and action of enzyme activators K^+ and acetyl-CoA. C249 is not involved in ATP binding as was observed for the corresponding residue in the biotin carboxylase subunit of *Escherichia coli* acetyl-CoA carboxylase, nor is it directly responsible for the measured inverse $^D(k_{cat}/K_m)$ isotope effects. The size of the inverse isotope effects indicates that they may result from formation of a low-barrier hydrogen bond. Modification of the wild type and C249A mutant with *o*-phthalaldehyde suggests that C249 is involved in isoindole formation but that the modification of this residue is not directly responsible for the accompanying major loss of enzyme activity.

Pyruvate carboxylase (EC 6.4.1.1) is a biotin-dependent enzyme that catalyzes the carboxylation of pyruvate to form oxaloacetate. The overall reaction that it catalyzes proceeds in two steps as follows:

$$\mathrm{MgATP} + \mathrm{HCO_3^-} + \mathrm{ENZ{-}biotin} \overset{\mathrm{Mg^{2+}, acetyl\text{-}CoA}}{\rightleftharpoons} \mathrm{MgADP} + \mathrm{P_i} + \mathrm{ENZ{-}biotin{-}CO_2^-} \quad (1)$$

$$\mathrm{ENZ{-}biotin{-}CO_2^-} + \mathrm{pyruvate} \rightleftharpoons \mathrm{ENZ{-}biotin} + \mathrm{oxaloacetate} \quad (2)$$

In reaction 1, the current evidence suggests that a carboxyphosphate intermediate is formed in a reaction between MgATP and HCO_3 (*1*, *2*). The biotin prosthetic group is then carboxylated at the 1′-N position either directly by carboxyphosphate or more likely by CO_2 resulting from the decarboxylation of the intermediate. The second partial reaction involves the carboxylation of pyruvate by carboxybiotin (reaction 2). Free Mg^{2+} and acetyl-CoA are only required in reaction 1, but the degree of dependence on acetyl-CoA for activity depends on the source of the enzyme (for a review, see ref *3*).

When it became apparent that the site of carboxylation of biotin was 1′-N, it was quickly realized that for this nitrogen to be sufficiently nucleophilic, the biotin must undergo tautomerization from the keto to the enol form (*4*). Tipton and Cleland (*5*) investigated the biotin carboxylase subunit of acetyl-CoA carboxylase from *Escherichia coli* and found evidence of a base−sulfhydryl ion pair which they proposed acted to enolize biotin. Tipton and Cleland (*5*) proposed that in the active form of the enzyme the base is protonated and the sulfhydryl group is deprotonated. A proton is removed from 1′-N of biotin by the ionized sulfhydryl group, and the protonated base stabilizes the negative charge of the ureido oxygen in the enolate tautomer of biotin. Later, Werneberg and Ash (*6*) reacted *o*-phthalaldehyde (*o*-Pa)[1] with chicken liver pyruvate carboxylase (CLPC) and demonstrated the formation of two isoindole derivatives per mole of biotin. The isoindole derivative is characteristic of the reaction of *o*-Pa with the ϵ-amino group of lysine and the sulfhydryl of cysteine when they are ~3 Å apart. From substrate protection

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[1] Abbreviations: Pyc1, pyruvate carboxylase I isozyme; Pyc2, pyruvate carboxylase 2 isozyme; WT, wild-type Pyc1; C249A, mutant form of Pyc1 in which C249 has been mutated to alanine; CLPC, chicken liver pyruvate carboxylase; *o*-Pa, *o*-phthalaldehyde.

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studies, it was proposed that there was one such amino acid pair present at the site of reaction 1. This work provided direct evidence that supported the involvement of a lysine−sulfhydryl ion pair in the carboxylation of biotin in a biotin-dependent enzyme.

Jitrapakdee *et al*. (*7*), when comparing the sequences of several pyruvate carboxylases with the crystal structure of the biotin carboxylase subunit of *E. coli* acetyl-CoA carboxylase determined by Waldrop *et al*. (*8*), noted that C230 and K238 are sufficiently close to allow their cross-linking by *o*-Pa and are located in the active site. These two residues are also strictly conserved in all biotin-dependent carboxylases, in a highly conserved region of amino acid sequence, i.e., ERD**C**SIQRRHQ**K**VVE. However, mutation of this cysteine to alanine in the biotin carboxylase subunit of acetyl-CoA carboxylase from *E. coli* (*9*) showed that C230 is not essential for the carboxylation of biotin. The mutation did result in a 50-fold increase in the K_m for MgATP, suggesting a role for this residue in ATP binding (*9*). The equivalent Cys in Pyc1 is C249.

Saccharomyces cerevisiae is the only organism known to have two genes for pyruvate carboxylase (*10*, *11*), namely, PYC1 and PYC2. The expression of the two genes is differentially regulated, and it has been proposed that different metabolic functions are carried out by the two isoforms of the enzyme (*12*, *13*). The sequences of the two isozymes, Pyc1 and Pyc2, are 92% homologous (*11*, *14*), and both are expressed in the cytoplasm (*15*). Studies on yeast pyruvate carboxylase to date have mainly been performed using the enzyme isolated from baker's yeast (*S. cerevisiae*) which has therefore potentially been a mixture of both of the isoenzymes. However, a preliminary study of the kinetic characteristics of Pyc1 has been published recently (*16*) and has shown that the properties of this isozyme differ from those previously reported for the apparently unresolved mixture of isoforms.

In this study, we have extended the kinetic characterization of Pyc1 to include a determination of its activation by K^+ and the first analysis of kinetic solvent deuterium isotope effects in reaction 1 of pyruvate carboxylase. We have produced a mutant form of the enzyme in which C249 has been mutated to alanine. In this way, we have examined the role of this residue in the mechanism of action of a biotin-dependent holoenzyme. Similar experiments have only been performed on one other member of the family of biotin-dependent carboxylases, and in that case, the isolated biotin carboxylase subunit of *E. coli* acetyl-CoA carboxylase was used (*9*).

We compare the results of our investigations of the biotin carboxylation reaction in the holoenzyme Pyc1 with those from studies of the isolated biotin carboxylase subunit of *E. coli* acetyl-CoA carboxylase.

MATERIALS AND METHODS

Strains and Vectors. A plasmid (pMW4A) containing the full-length, wild-type *S. cerevisiae* PYC1 gene (*17*) in pUC19 was kindly provided by M. Walker (Biochemistry Department, University of Adelaide). The Pyc1 mutant and the wild-type control were cloned into the pVT100-U multicopy yeast expression vector (*18*) and transformed into *S. cerevisiae* DM18 (MATα, *ura3*, *trp1*, *pyc1*, LEU2, *pyc2*, HIS3) (*13*).

Site-Directed Mutagenesis. A 0.5 kb *Kpn*I−*Bgl*II fragment encoding part of the biotin carboxylase domain of Pyc1 was restricted from pMW4A and ligated into pBluescript II SK (±) (Stratagene). Mutagenesis was performed on this fragment using the Quickchange site-directed mutagenesis kit (Stratagene). The mutagenic oligonucleotides for C249A are 5′-CGAAAGAGAC**GCT**AGCGTGCAGAGAAGAC-3′ (forward) and 5′-GTCTTCTCTGCACGCT**AGC**GTCTCTTTCG-3′ (mutated codons are in bold, and a silent *Nhe*I restriction site is underlined). The mutant was screened by *Nhe*I restriction digestion and sequenced to confirm the mutation. After mutagenesis, this 0.5 kb *Kpn*I−*Bgl*II cassette was used to reassemble the PYC1 gene in pVT100-U.

Transformation of Mutant Expression Constructs into Yeast. Yeast transformation was performed using the lithium acetate procedure (*19*). Transformants were plated onto uracil-deficient selective media [0.8% (w/v) yeast nitrogen base, 1.1% (w/v) casamino amino acids, 0.2 g/L adenine, 2% (w/v) glucose, 0.01% (w/v) tryptophan, 0.01% (w/v) leucine, 2% (w/v) bactoagar, and 0.05% (w/v) D-biotin], supplemented with 12 mM L-aspartate, and incubated at 30 °C for 6 days to reveal transformant colonies.

Preparation and Purification of WT and C249A. The WT and C249A enzymes were purified as described by Branson *et al*. (*16*).

Other Materials. [^{14}C]Biotin was obtained as a solid at a specific radioactivity of 50−62 mCi/mmol from Amersham-Pharmacia, Australia Pty. Ltd. All other materials were high-purity preparations from commercial suppliers.

Assays of Pyruvate Carboxylating Activity. The conditions for this spectrophotometric assay where oxaloacetate formation is assessed in a coupled reaction with malate dehydrogenase, were as described by Attwood and Cleland (*20*), except that the buffer was 0.1 M Tris-HCl (pH 7.8). One unit of enzyme activity is defined as the amount of enzyme required to catalyze the formation of 1 μmol of oxaloacetate per minute at 30 °C. Assays were performed at 30 °C with the following concentrations of substrates unless that substrate was varied: 2.5 mM ATP, 20 mM $NaHCO_3$, 7 mM $MgCl_2$, 10 mM pyruvate, and 0.21 mM NADH. Unless varied or stated otherwise, acetyl-CoA and K^+ concentrations were 0.25 and 5.1 mM, respectively, for WT and 1 and 20.5 mM, respectively, for C249A. In these assays, the concentrations of acetyl-CoA that were present gave comparable saturations of WT (81%) and C249A (78%), as did the concentrations of K^+: 57% for WT and 59% for C249A. In the experiments where the K^+ concentration was varied, no acetyl-CoA was present, and in the case where the acetyl-CoA concentration was varied, no K^+ was present (acetyl-CoA was synthesized from the sodium salt of CoASH by reaction with acetic anhydride in the presence of $NaHCO_3$ instead of $KHCO_3$).

ADP Phosphorylation by Carbamoyl Phosphate. These assays were performed at 30 °C as described by Attwood and Graneri (*21*), using hexokinase and glucose-6-phosphate dehydrogenase as coupling enzymes to assess MgATP formation. The sodium salt of carbamoyl phosphate was used as the varied substrate, and 2 mM MgADP was present, in the presence or absence of acetyl-CoA and K^+ at concentrations for pyruvate carboxylation assays of WT and C249A as described above.

ATPase Reactions in the Absence of Pyruvate. These assays were performed at 30 °C as described by Attwood and Graneri (*20*), using pyruvate kinase and lactate dehydrogenase as coupling enzymes to assess MgADP formation. MgATP was the varied substrate, and reactions were performed in the presence of 20 mM $NaHCO_3$. When present, acetyl-CoA and K^+ concentrations were as described above for pyruvate carboxylation assays of WT and C249A.

Solvent Deuterium Isotope Effects. The solvent deuterium isotope effects on both the full pyruvate carboxylation reaction, the ADP phosphorylation reaction, and the ATPase reaction in the absence of pyruvate were determined as described above except that the reactions were performed in 95 or 97% D_2O as indicated. The reactions for the proton inventories were performed in reaction mixtures containing 0−95% D_2O using 0.25 mM (WT) or 4.6 mM (C249A) K^+-free acetyl-CoA.

Determination of the Biotin Content. After removal from the storage solution, aliquots of the enzyme solutions were set aside and stored at −80 °C for later determination of the biotin content. The enzyme solutions were incubated with 0.2% (w/v) chymotrypsin at 37 °C for 24 h. Pronase was then added to a final concentration of 0.45% (w/v), and the solutions were incubated for an additional 72 h at 37 °C. The solutions were then heated for 15 min at 100 °C before being used in the biotin assay described by Rylatt *et al*. (*22*).

Modification of WT and C249A by o-Pa. This was performed at 30 °C as described by Werneberg and Ash (*6*) using 100 μM *o*-Pa. The number of isoindoles formed per enzymic subunit were calculated from the isoindole absorbance at 337 nm at various time points over the 15 min modification time course and from the biotin concentration of the enzyme. An absorbance coefficient of 7660 M^{-1} cm^{-1} at 337 nm (*6*) was used to calculate the isoindole concentrations.

Data Analysis. The data were analyzed using nonlinear least-squares regression analysis to fit the data either to the Michaelis−Menten equation or in the cases where the effector acetyl-CoA or K^+ was varied to

$$v = V_{max}/(1 + K_a/[\text{effector}]) + R \qquad \text{(i)}$$

where v is the velocity of the reaction at any effector concentration ([acetyl-CoA] or [K^+]), V_{max} is the velocity of the reaction at a saturating acetyl-CoA or K^+ concentration, K_a is equal to the acetyl-CoA or K^+ concentration required to give $v = V_{max}/2$, and R is the residual velocity of the reaction in the absence of acetyl-CoA or K^+. Pseudo-first-order rate constants were estimated together with standard errors of the estimates for the inactivation of the enzyme with *o*-Pa by fitting first-order exponential decay curves to the data.

RESULTS

Kinetic Parameters of WT and C249A for the Full Pyruvate Carboxylation Reaction. K_a values for acetyl-CoA and K^+ and K_m values for bicarbonate, MgATP, and pyruvate for the full pyruvate carboxylation reaction catalyzed by both WT and C249A are shown in Table 1. In addition, k_{cat} values for the reaction in the presence of a saturating acetyl-CoA or saturating K^+ concentration are shown. The K_a for acetyl-CoA of C249A was 4.8-fold greater than that of WT, while the effect of the mutation on the K_a for K^+ was to increase it by 3.7-fold from that of WT. Essentially, the K_m values for MgATP and pyruvate were unaffected by the mutation. The K_m for bicarbonate of C249A was 1.7-fold greater than that of WT.

Table 1: Kinetic Parameters for the Full Pyruvate Carboxylation Reaction of WT and C249A[a]

	K_m or K_a (±SE) (mM)	k_{cat} (±SE) (s^{-1})
AcCoA		
WT	0.06 ± 0.01	59.5 ± 1.9
C249A	0.29 ± 0.03	20.1 ± 0.8
MgATP		
WT	0.07 ± 0.01	
C249A	0.05 ± 0.02	
HCO_3^-		
WT	1.36 ± 0.12	
C249A	2.30 ± 0.39	
pyruvate		
WT	0.50 ± 0.06	
C249A	0.45 ± 0.08	
K^+		
WT	3.80 ± 0.62	29.9 ± 1.2
C249A	14.02 ± 3.25	2.94 ± 0.34

[a] The K_m values of WT for the substrates are taken from ref *16* and were determined using acetyl-CoA containing K^+, and hence, the assays contained 0.25 mM acetyl-CoA and 5.1 mM K^+ (see Materials and Methods). The K_m values of C249A were determined in the presence of 1 mM acetyl-CoA and 20.5 mM K^+. The K_a values for acetyl-CoA and k_{cat} values in the presence of a saturating acetyl-CoA concentration for WT and C249A were determined in the absence of K^+. The K_a values for K^+ and the k_{cat} values in the presence of a saturating K^+ concentration for WT and C249A were determined in the absence of acetyl-CoA. The standard errors (SE) of the estimates of the kinetic parameters were derived from the nonlinear least-squares fits of the Michaelis−Menten equation to the data or in the case of acetyl-CoA and K^+ data to eq i.

The k_{cat} for the reaction in the presence of saturating concentrations of acetyl-CoA of WT was approximately twice that in the presence of a saturating K^+ concentration. The mutation of C249 resulted in a k_{cat} that was ∼34% of that of WT for the reaction in the presence of a saturating acetyl-CoA concentration. In the presence of a saturating K^+ concentration, however, the k_{cat} for the reaction catalyzed by C249A was only ∼10% of that of that of WT. In addition, the k_{cat} for the reaction in the absence of acetyl-CoA was 7.4% of that in the presence of a saturating acetyl-CoA for WT concentration but not significantly different from zero for C249A (data not shown).

Kinetic Parameters of WT and C249A for ADP Phosphorylation by Carbamoyl Phosphate. The kinetic parameters for ADP phosphorylation by carbamoyl phosphate are shown in Table 2. In the reaction catalyzed by WT, acetyl-CoA and K^+ had little effect on either k_{cat} or K_m for carbamoyl phosphate. The C249A mutation only affected k_{cat} and K_m in the presence of acetyl-CoA and K^+, where k_{cat} was reduced by 60% and K_m by 85%, and this resulted in a 2.6-fold increase in k_{cat}/K_m compared to that of WT.

Solvent Deuterium Isotope Effects. The kinetic solvent deuterium isotope effects for the full carboxylation reaction, the ATPase reaction in the absence of pyruvate, and the phosphorylation of ADP by carbamoyl phosphate are shown in Table 3. The $^D(k_{cat}/K_m)$ effects for the full pyruvate carboxylation reaction for both WT and C249A were inverse effects, in both the presence and absence of acetyl-CoA and K^+. The $^D(k_{cat}/K_m)$ effects for WT and C249A were much

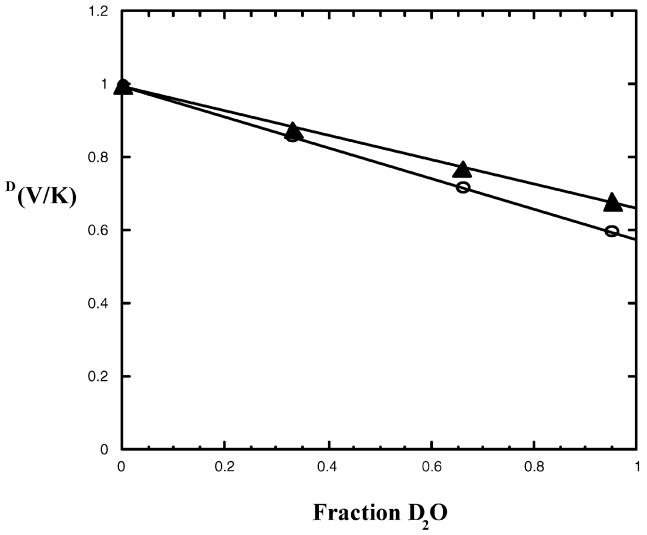

FIGURE 1: Proton inventories for the full pyruvate carboxylation reaction in WT (▲) and C249A (○) with MgATP as the varied substrate. Other reaction components were pyruvate (10 mM), $NaHCO_3$ (20 mM), free Mg^{2+} (4.5 mM), and acetyl-CoA (0.25 mM for WT or 4.6 mM for C249A).

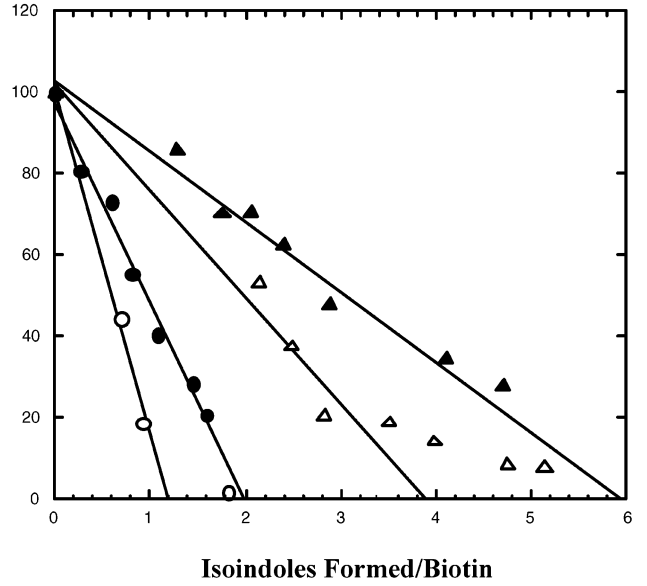

FIGURE 2: Stoichiometry of modification of WT (△) and the C249A mutant (○) by *o*-Pa, in the absence (empty symbols) or presence (filled symbols) of acetyl-CoA and K^+. Where present, the reaction mixtures contained either 0.25 mM acetyl-CoA and 5.1 mM K^+ (WT) or 1 mM acetyl-CoA and 20.5 mM K^+ (C249A).

more inverse in the absence of acetyl-CoA and K^+ than in their presence, and that for C249A was more inverse than for WT in the absence of acetyl-CoA and K^+. The $^Dk_{cat}$ effect was an inverse effect in the absence of acetyl-CoA and K^+ for both WT and C249A, while in their presence, the effect was small and normal for WT and not significantly different from unity for C249A. Figure 1 shows proton inventories for the solvent $^D(k_{cat}/K_m)$ effects for both WT and C249A, and the linearity of both plots indicates the involvement of a single proton transfer step.

In the ATPase reaction, in both the presence and absence of acetyl-CoA and K^+, WT exhibited $^D(k_{cat}/K_m)$ effects that were inverse and similar to that observed for the full pyruvate carboxylation reaction in the presence of acetyl-CoA and K^+. C249A too had an inverse $^D(k_{cat}/K_m)$ effect in the absence of acetyl-CoA and K^+ similar in size to that of WT. However, in the presence of acetyl-CoA and K^+, the $^D(k_{cat}/K_m)$ effect for C249A was large and normal. All $^Dk_{cat}$ effects were close to 1.

Table 2: Kinetic Parameters for the ADP Phosphorylation by Carbamoyl Phosphate[a]

	K_m (±SE) (mM)	k_{cat} (±SE) (s^{-1})
with AcCoA and K^+		
WT	11.3 ± 2.3	0.11 ± 0.01
C249A	1.7 ± 0.4	0.043 ± 0.003
without AcCoA and K^+		
WT	12.3 ± 3.8	0.11 ± 0.01
C249A	10.7 ± 4.5	0.13 ± 0.02

[a] The kinetic parameters were determined by varying the carbamoyl phosphate concentration at 2 mM MgADP in the presence or absence of either 0.25 mM acetyl-CoA and 5.1 mM K^+ (WT) or 1 mM acetyl-CoA and 20.5 mM K^+ (C249A). The standard errors (SE) of the estimates of the kinetic parameters were determined from nonlinear least-squares fits of the Michaelis−Menten equation to the data.

In the ADP phosphorylation reaction, in the presence of acetyl-CoA and K^+, WT exhibited a $^D(k_{cat}/K_m)$ effect that was inverse and similar to those for the full pyruvate carboxylation reaction and the ATPase reaction in the presence of acetyl-CoA and K^+. The $^Dk_{cat}$ effect was close to 1 for WT. In contrast, C249A exhibited $^D(k_{cat}/K_m)$ and $^Dk_{cat}$ effects that were both normal and similar in magnitude to each other.

Modification of WT and C249A by o-Pa. Figure 2 shows the stoichiometry of modification by *o*-Pa of the WT and C249A mutant enzymes. The intersection points of the lines (fitted to the initial data points of each data set) on the abscissa indicate that more isoindole derivatives are formed during the observed inactivation process in WT than in C249A. On the other hand, the pseudo-first-order rate constants measured for the inactivation of the enzyme were similar for both WT and C240A in the absence of acetyl-CoA and K^+: 0.0100 ± 0.0020 s^{-1} for WT and 0.0066 ± 0.0026 s^{-1} for C249A. The values were 0.0022 ± 0.0017 and 0.0025 ± 0.0011 s^{-1}, respectively, in their presence. As well as reducing the rate of inactivation, acetyl-CoA and K^+ also increased the number of isoindole derivatives formed at each level of residual activity.

DISCUSSION

In WT, the K_a for K^+ is ∼45% of that measured by Myers *et al*. (23) in baker's yeast. The presence in the baker's yeast of Pyc2, which may have a higher K_a for K^+, is likely to account for this difference. This, together with previously published data which suggested differences between Pyc1 and Pyc2 in their K_a values for acetyl-CoA (*15*), indicates that a major difference between the two isozymes is their regulation by allosteric activators. It should be noted that in the work presented here K^+-free acetyl-CoA was used whereas previously (*15*) acetyl-CoA that did contain K^+ was used. The values thus reported in the work presented here of the K_a for acetyl-CoA and the k_{cat} in the presence of a saturating acetyl-CoA concentration for WT are somewhat smaller than those previously reported.

The lack of an effect of acetyl-CoA and K^+ on the kinetic parameters of the ADP phosphorylation reaction in WT differs markedly from the effect for the same reaction in

Table 3: Solvent Deuterium Isotope Effects, $^{D}(k_{cat}/K_m)$, and $^{D}k_{cat}$ on the Full Pyruvate Carboxylation Reaction, the ATPase Reaction in the Absence of Pyruvate, and the ADP Phosphorylation by Carbamoyl Phosphate[a]

	$WT_{+AcCoA+K}$ (±SE)	$WT_{-AcCoA+K}$ (±SE)	$C249A_{+AcCoA+K}$ (±SE)	$C249A_{-AcCoA+K}$ (±SE)
	(A) Full Pyruvate Carboxylation Reaction			
$^{D}(k_{cat}/K_m)$	0.64 ± 0.04	0.33 ± 0.02	0.56 ± 0.03	0.13 ± 0.02
$^{D}k_{cat}$	1.46 ± 0.08	0.61 ± 0.04	1.02 ± 0.09	0.58 ± 0.03
	(B) ATPase Reaction			
$^{D}(k_{cat}/K_m)$	0.67 ± 0.05	0.76 ± 0.12	3.75 ± 0.46	0.60 ± 0.09
$^{D}k_{cat}$	1.12 ± 0.03	0.91 ± 0.05	1.01 ± 0.07	1.26 ± 0.09
	(C) ADP Phosphorylation by Carbamoyl Phosphate			
$^{D}(k_{cat}/K_m)$	0.66 ± 0.05	−	1.48 ± 0.35	−
$^{D}k_{cat}$	1.05 ± 0.07	−	1.55 ± 0.26	−

[a] Isotope effects were determined in either 95% (A) or 97% D_2O (B and C) by varying the MgATP concentration while holding the excess Mg^{2+} concentration at 4.5mM for the full pyruvate carboxylation and ATPase reactions. The carbamoyl phosphate concentration was varied in the ADP phosphorylation reaction. All other substrates were present as described in Materials and Methods, and reactions were performed in the presence or absence of either 0.25 mM acetyl-CoA and 5.1 mM K^+ (WT) or 1 mM acetyl-CoA and 20.5 mM K^+ (C249A). The standard errors (SE) are of the means of three estimates.

chicken liver pyruvate carboxylase (CLPC). Although both enzymes are very dependent on acetyl-CoA for overall pyruvate carboxylating activity, acetyl-CoA induced a more than 200-fold increase in the size of the observed rate constant of the approach to steady state of ATP cleavage in CLPC (*24*), but had no effect in WT (*16*). ADP phosphorylation by carbamoyl phosphate is thought to mimic the reverse of the ATP cleavage and carboxyphosphate formation, and thus, the large difference in the effects of acetyl-CoA on the ATP cleavage reaction in WT and CLPC appears to be reflected in the ADP phosphorylation reaction.

Solvent D_2O isotope effects have not been previously measured for the full pyruvate carboxylation reaction, the ATPase reaction in the absence of pyruvate, or the phosphorylation of ADP by carbamoyl phosphate in pyruvate carboxylase. However, solvent D_2O isotope effects have been measured for these reactions catalyzed by the biotin carboxylase subunit of *E. coli* acetyl-CoA carboxylase (*5*, *9*). For these reactions in biotin carboxylase, inverse solvent D_2O isotope effects were observed on both k_{cat} and k_{cat}/K_m (*5*, *9*) and were attributed to a single proton transfer step (*5*). Our results for WT are similar for the isotope effects on k_{cat}/K_m in the full pyruvate carboxylation reaction, the ATPase reaction in the absence of pyruvate, and the ADP phosphorylation reaction. In addition, the linearity of the proton inventory for WT shown in Figure 1 indicates that the effects in WT can be attributed to a single proton transfer step. In contrast to the isotope effects in biotin carboxylase, however, an inverse $^{D}k_{cat}$ effect was only observed for the full pyruvate carboxylation reaction when acetyl-CoA and K^+ were absent. In the other reactions, $^{D}k_{cat}$ was either close to unity or slightly normal. This suggests that while the isotope-sensitive step is rate-limiting for both k_{cat}/K_m and k_{cat} in all of the reactions in biotin carboxylase this is the only case for the full pyruvate carboxylation reaction in the absence of acetyl-CoA and K^+ in WT. In all the other cases in WT, isotope-insensitive reaction steps, which may not occur in the biotin carboxylase reaction, are rate-limiting for k_{cat}.

The overall similarity of the results suggests that the cause of the inverse isotope effects is the same isotope-sensitive step in both WT and biotin carboxylase. Tipton and Cleland (*5*) interpreted the fact that the isotope effects on both k_{cat}/K_m and k_{cat} for biotin carboxylation and ADP phosphorylation were inverse effects in both cases as an indication that biotin carboxylase exists in two protonation states, only one of which is catalytically active. Thus, for ATP cleavage, for biotin carboxylation, and for ADP phosphorylation by carbamoyl phosphate, the inactive form of the enzyme has to convert to the active form in a deuterium-sensitive proton transfer reaction. Largely on the basis of what was known of deuterium isotope fractionation factors at the time and from their experiments with sulfhydryl group-modifying reagents, Tipton and Cleland (*5*, *24*) suggested that the reaction responsible for the isotope effects was proton transfer from a cysteine sulfhydryl group to the amino group of a lysine residue, resulting in the catalytically active form of the enzyme. In the biotin carboxylation reaction, the resultant ionized sulfhydryl and protonated lysine amine then act in concert to induce enolization of the biotin, thus activating the 1′-N ready for carboxylation (*5*). Since 1988, however, it has become apparent that low deuterium isotope fractionation factors can also be indicative of the presence of a low-barrier hydrogen bond (*25*). In addition, the values of $^{D}(k_{cat}/K_m)$ we have obtained for the full pyruvate carboxylation reaction of 0.33 in WT and 0.13 in C249A, together with values of 0.21−0.32 for the ATPase reaction of biotin carboxylase (*9*), are much lower than the fractionation factor of sulfhydryl groups of 0.4−0.5 (*26*). This suggests that the formation of one or more low-barrier hydrogen bonds may be responsible for the observed solvent deuterium isotope effects. It is likely that that the formation of the putative low-barrier hydrogen bond occurs as a result of proton rearrangements in the active site that are associated with the enolization of biotin and its subsequent carboxylation. At this stage, however, we cannot identify the amino acid residues involved in the formation of the low-barrier hydrogen bond nor understand its detailed role in the catalysis of biotin carboxylation.

In general, the mutation of C249 produced small but significant effects on some kinetic parameters of Pyc1 which suggests that while C249 does not play an essential role in catalysis, it does participate in certain aspects of enzyme function. The mutation of C249 had little effect on the K_m for pyruvate, indicating that no large structural changes had occurred that affected the site of reaction 2 (Table 1). More unexpectedly, however, the mutation also did not affect the K_m for MgATP (Table 1). This is in stark contrast to the more than 50-fold increase in K_m that the corresponding

C230A mutation in the biotin carboxylase subunit of *E. coli* acetyl-CoA carboxylase produced which Levert *et al*. took as an indication of a role in ATP binding for C230 (*9*). It is possible that the isolated biotin carboxylase subunit has a somewhat different conformation compared to that when it is part of the acetyl-CoA carboxylase holoenzyme, a circumstance under which its structure may be more similar to the biotin carboxylation domain of pyruvate carboxylase. This might be inferred by the more than 6000-fold increase in catalytic efficiency of biotin carboxylase with an 87-amino acid C-terminal domain of the biotin carboxyl carrier protein of *E. coli* acetyl-CoA carboxylase (BCCP87) as a substrate, compared to the reaction with free biotin as a substrate (*27*). With BCCP87 as a substrate, the reaction and conformation of the biotin carboxylase subunit are likely to be closer to those that occur in the acetyl-CoA carboxylase holoenzyme than to those with free biotin as a substrate.

The major effects of the C249 mutation in Pyc1 are on the stimulation of enzyme activity by acetyl-CoA and K^+ and the abolition of enzyme activity in the absence of these activators. The effects of the mutation of C249 on both K_a and k_{cat} of the activation of Pyc1 by acetyl-CoA and K^+ indicate that C249 is involved in both the binding of these activators and mediating their activation of catalysis. The other effect of the C249 mutation is to increase the K_m of HCO_3^-; however, this may not be as a result of a direct effect of the mutation on HCO_3^- binding but may be a result of the reduced efficacy of activation of the enzyme by acetyl-CoA, since one effect of acetyl-CoA is to reduce the K_m for HCO_3^- (*28*).

In the ADP phosphorylation reaction, the mutation had no effect on the K_m for carbamoyl phosphate or k_{cat} in the absence of acetyl-CoA and K^+ but reduced both K_m and k_{cat} in their presence, resulting in an increase in catalytic efficiency of ~2.7-fold compared to that of WT. These effects of the C249 mutation on the ADP phosphorylation reaction are qualitatively similar to those measured by the mutation of C230 in biotin carboxylase (*9*).

In C249A, the solvent $^D k_{cat}$ isotope effects on all of the reactions are similar to those observed in WT and indicate that the mutation is not having a great effect on the reaction step(s) which is rate-limiting for k_{cat}. In C249A, the solvent $^D(k_{cat}/K_m)$ isotope effects for the full pyruvate carboxylation reaction are similar to those of WT. However, the $^D(k_{cat}/K_m)$ effects for ADP phosphorylation and the ATPase reaction in the presence of acetyl-CoA and K^+ are normal and large in the case of the ATPase reaction. In their study of the ATPase reaction in Pyc1, Branson *et al*. (*16*) found evidence which suggested that acetyl-CoA enhances the rates of ATP cleavage and movement of biotin between the sites of reactions 1 and 2. In addition, acetyl-CoA also caused a decrease in the extent of coupling between ATP cleavage and carboxybiotin formation such that abortive hydrolysis of carboxyphosphate becomes the major pathway in the catalytic cycle, as opposed to biotin carboxylation. The observed normal solvent $^D(k_{cat}/K_m)$ effect observed in C249A in the presence of acetyl-CoA could arise as a result of this redirection of flux through the catalytic cycle. One possibility is that the mutation of C249 results in this hydrolysis reaction, with an attendant normal deuterium isotope effect, becoming rate-limiting for k_{cat}/K_m. In the ADP phosphorylation reaction, it would appear that in C249A a deuterium-insensitive step (or at least one with only a small normal isotope effect) has become rate-limiting for both k_{cat}/K_m and k_{cat}. The marked effects of the mutation of C249 on the K_m for carbamoyl phosphate and the k_{cat} for the reaction (Table 2) support this proposal. Since there are no proton transfer reactions in this reaction, the new rate-limiting step may involve product dissociation.

The modification of WT by *o*-Pa differs from that of CLPC observed by Werneberg and Ash (*6*), who found that the loss of enzymic activity correlated to the formation of approximately 1.3 isoindoles per biotin, although more prolonged incubation gave evidence of the formation of multiple isoindole derivatives per biotin. In WT, this stoichiometry is closer to 4 isoindoles per biotin in the absence of acetyl-CoA and 6 in its presence. CLPC has 15 Cys and 37 Lys residues (*29*), whereas Pyc1 has 12 Cys and 65 Lys residues; thus, although Pyc1 has fewer Cys residues than CLPC, it is possible that the larger number of Lys residues in Pyc1 creates more Cys-Lys pairs in the proximity of each other which can be modified by *o*-Pa. Differences in the numbers and spatial arrangements of Cys and Lys residues between Pyc1 and Pyc2 may also explain the finding of Headlam and Attwood (*30*) that for baker's yeast PC the stoichiometry of isoindole formation per biotin correlating to the loss of enzymic activity was close to 1.

The increase in the stoichiometry of isoindole formation per biotin during inactivation and the 4−5-fold reduction in the observed first-order rate constant for inactivation in the presence of acetyl-CoA and K^+ are indications of the protection against *o*-Pa inactivation of the enzyme afforded by these effectors.

The C249A mutation results in less isoindole formation at a corresponding level of loss of enzyme activity compared to that for WT upon modification of the enzyme by *o*-Pa, with stoichiometries of isoindole formation per biotin of 1.2 and 2 in the absence and presence of acetyl-CoA, respectively. This indicates that in WT, C249 is modified by *o*-Pa, probably together with K257, to form an isoindole. The fact that stoichiometries of isoindole formation in C249A are much lower than those of WT indicates that the mutation has also affected the *o*-Pa modification of other Cys-Lys pairs, apart from C249 and K257. On the other hand, the observed first-order rate constants for enzyme inactivation in C249A are very similar to those of WT. This suggests that the major part of the loss of enzyme activity on modification of the enzyme with *o*-Pa is not caused by isoindole formation involving C249 but is due to isoindole formation involving another Cys-Lys pair.

This work has provided more evidence that the major difference between the two isozymes of pyruvate carboxylase in *S. cerevisiae* (Pyc1 and Pyc2) lies in their allosteric regulation. The solvent deuterium isotope effects measured have indicated that a single proton transfer step is responsible and that it converts the enzyme from a catalytically inactive to an active form. The size of inverse isotope effects observed under some conditions indicates that formation of a low-barrier hydrogen bond occurs and, not as previously thought, proton transfer from a sulfhydryl group occurs. The fact that C249 has been shown not to be responsible for the loss of activity during *o*-Pa modification of the enzyme and to be nonessential for catalysis and the fact that similar isotope effects still occur in the full pyruvate carboxylation reaction

in C249A compared to WT rule out the originally proposed role of this residue in the mechanism of the enzyme. However, C249 has been shown to be involved in the binding and activation of the enzyme by allosteric activators acetyl-CoA and K^+. C249 does not appear to have a role in ATP binding, unlike C230 in biotin carboxylase (*8*), and this may be due to differences between the structure of the isolated biotin carboxylase subunit of acetyl-CoA carboxylase and that of the biotin carboxylase domain in the pyruvate carboxylase holoenzyme, despite their ~44% level of sequence identity.

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