Pergamon

Bioorganic & Medicinal Chemistry 7 (1999) 887–894

BIOORGANIC & MEDICINAL CHEMISTRY

The Linkage of Catalysis and Regulation in Enzyme Action: Oxidative Diversion in the Hysteretically Regulated Yeast Pyruvate Decarboxylase

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Received 24 September 1998; accepted 17 November 1998

Abstract—The reaction catalyzed by the thiamin-diphosphate-dependent yeast pyruvate decarboxylase, which is hysteretically regulated by pyruvate, undergoes paracatalytic oxidative diversion by 2,6-dichlorophenolindophenol, which traps a carbanionic intermediate and diverts the product from acetaldehyde to acetate (Christen, P. *Meth. Enzymol.* **1977**, *46*, 48). This reaction is now shown to exhibit an oxidant on-rate constant somewhat faster than that for pyruvate in the normal catalytic cycle and a product off-rate constant about 60-fold smaller than that for acetaldehyde. Both on-rates and off-rates exhibit an inverse solvent isotope effect of 1.5-2, observed in normal catalysis as a signal of sulfhydryl addition to the keto group of pyruvate at the allosteric regulatory site. The findings are consistent with a model for regulation in which the sulfhydryl-addition process mediates access to a fully catalytically competent active site, the oxidative-diversion reaction being forced to make use of the normal entry–exit machinery.

Introduction

The interface of chemistry and biology is a region of which Professor Sir Derek H. R. Barton was a pioneer explorer and a region of which he was a comfortable and influential inhabitant. His major instrument of exploration was the explanation of biological phenomenology in the rigorous language of chemistry, commonly at the level of molecular structure and often at the submolecular level. In this paper, which we dedicate respectfully to his memory, we attempt on a scale very modest by Barton standards a chemical examination of features linking catalysis and regulation in enzyme action.

Yeast pyruvate decarboxylase[1]

The pyruvate decarboxylase (EC 4.1.1.1) of the yeast *Saccharomyces cerevisiae* (SCPDC) is a thiamin-diphosphate (ThDP)-dependent enzyme that catalyzes the conversion of pyruvic acid to carbon dioxide and acetaldehyde (Fig. 1). Its thoroughly investigated catalytic mechanism and its striking regulatory properties make it an effective system in which to explore how catalysis and regulation are linked in enzyme action. The yeast is capable in the presence of oxygen of the oxidative metabolism of carbohydrates through the tricarboxylic-acid cycle and the electron-transport chain, a metabolic pathway that produces a high yield of energy stored in adenosine triphosphate. The organism is also capable in the absence of oxygen of fermentative metabolism to produce a lower energy yield. SCPDC occupies the branch point between these metabolic routes and, perhaps as a result, is regulated by its own substrate in an unusual hysteretic, or time-dependent, manner. The regulatory action may protect the organism from losses of energy that would occur if pyruvate from glycolysis were partially diverted into the fermentative pathway when oxygen is available for the more productive electron-transport pathway.

The hysteretic regulatory feature of SCPDC renders the enzyme completely inactive upon initial contact with the substrate pyruvate (i.e., the asymptotic rate of product formation is zero at the time of exposure to pyruvate). In a period of typically 10–30 seconds, the rate of product formation relaxes to the steady-state value. The rate of relaxation is saturable in pyruvate. The steady-state velocity is second order in pyruvate at low concentrations, reflecting the requirement for the enzyme first to combine with a 'regulatory pyruvate' and then with a 'catalytic pyruvate'. At higher concentrations as the regulatory interaction becomes saturated, the steady-state velocity becomes first order in pyruvate and, finally, when the catalytic reaction becomes saturated, the rate becomes zero order in pyruvate. Surrogate regulators that possess a carbonyl group but do not

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0968-0896/99/$ - see front matter
PII: S0968-0896(98)00269-7

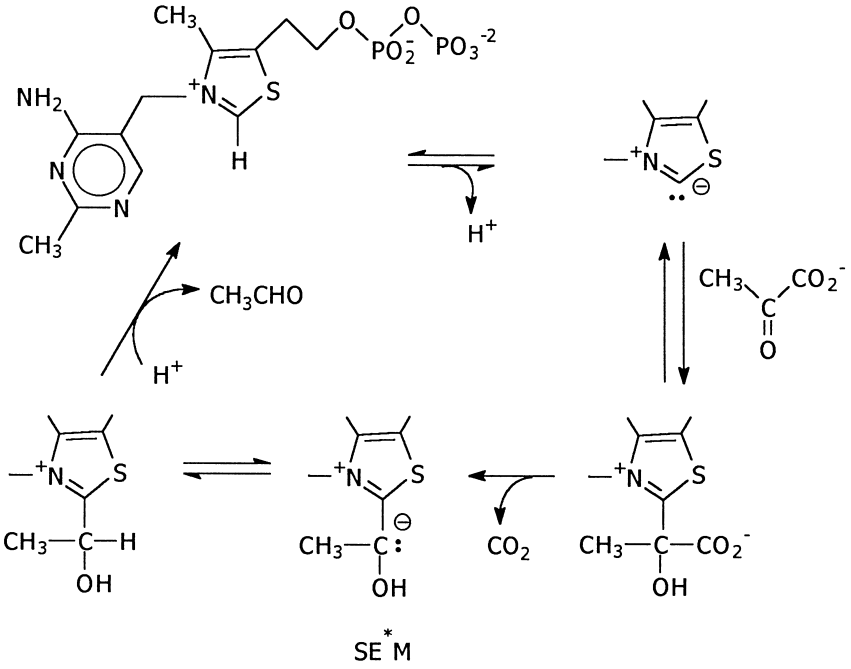

Figure 1. The normal catalytic cycle for SCPDC. Note the structure of thiamin diphosphate (ThDP) with a diphosphate arm and a pyrimidine arm. Ionization of the acidic C2–H bond leads to the 'first carbanion'. Addition of this species to the substrate pyruvate produces decarboxylation and formation of the 'second carbanion'. Protonation of the latter and elimination to form acetaldehyde completes the catalytic cycle. The 'second carbanion' is denoted SE*M in the system[20] used herein for mechanistic descriptions (see Figs 2 and 5).

undergo decarboxylation (pyruvamide and ketomalonate) can replace pyruvate in the regulatory interaction. The steady-state velocity is then hyperbolically dependent on pyruvate.

Catalysis by SCPDC[1–3]

Catalysis by yeast pyruvate decarboxylase is typical of ThDP-dependent decarboxylases (Fig. 1). The enzyme possesses three domains.[4] A diphosphate-binding domain and a pyrimidine-binding domain serve to anchor the cofactor with its thiazolium C_2–H bond directed toward the presumed pyruvate binding site. The third domain is known as the regulatory domain; we will return to it below. Ionization of the C_2–H bond leads to addition across the pyruvate carbonyl group. In the adduct, fission of the C–C bond in decarboxylation is promoted by electron delocalization into the thiazolium nucleus. The 'second carbanion' formed as the first product of decarboxylation is then protonated. An elimination reaction produces the product acetaldehyde and regenerates the cofactor.

Regulation of SCPDC[5–7]

Hysteretic regulation of pyruvate is critically dependent[8] on the residue Cys-221, which is located in the regulatory domain about 2 nm from the thiazolium nucleus. When this residue is mutated to serine, about 15% of the enzyme activity remains but the kinetics become simply hyperbolic and the initial relaxation period disappears: the enzyme has become unregulated. Some natural pyruvate decarboxylases are also unregulated, displaying hyperbolic kinetics and no initial relaxation period. One of these is the pyruvate decarboxylase of the bacterium *Zymomonas mobilis*,[9–12] in which a cysteine residue is not present at the position corresponding to Cys-221 in SCPDC. Earlier work had already implicated sulfhydryl groups in regulation.[13]

Surrogate regulators of SCPDC can replace the 'regulatory pyruvate', as already mentioned. All such species possess a carbonyl group and this fact, together with the strong evidence for the role of Cys-221 have led to the concept that at some point in the regulatory process, the sulfhydryl group of Cys-221 adds to the carbonyl group of the regulatory molecule.

Models for PDC regulation: active-site reorganization

Jordan, Furey, and their co-workers have made use of mutagenesis experiments[14] and of the crystal structure[4] to propose a model for the regulatory activation of SCPDC. They employ the consensus hypothesis that addition to the keto group of pyruvate by Cys-221-SH is important, and they further suppose that this is the event that triggers activation of the enzyme. They have shown that residues that form a network of interactions reaching from Cys-221 to the active site are important for activation and they therefore propose that the network transfers the information to the active site that the activation event has occurred, the resulting active-site reorganization then giving rise to full catalytic activity.

Models for PDC regulation: active-site access

We have favored a different model,[15,16] illustrated in Figure 2. According to this model, the time-dependent activation represents a 'seating' of the regulatory pyruvate, perhaps by coordination of its carboxylate group

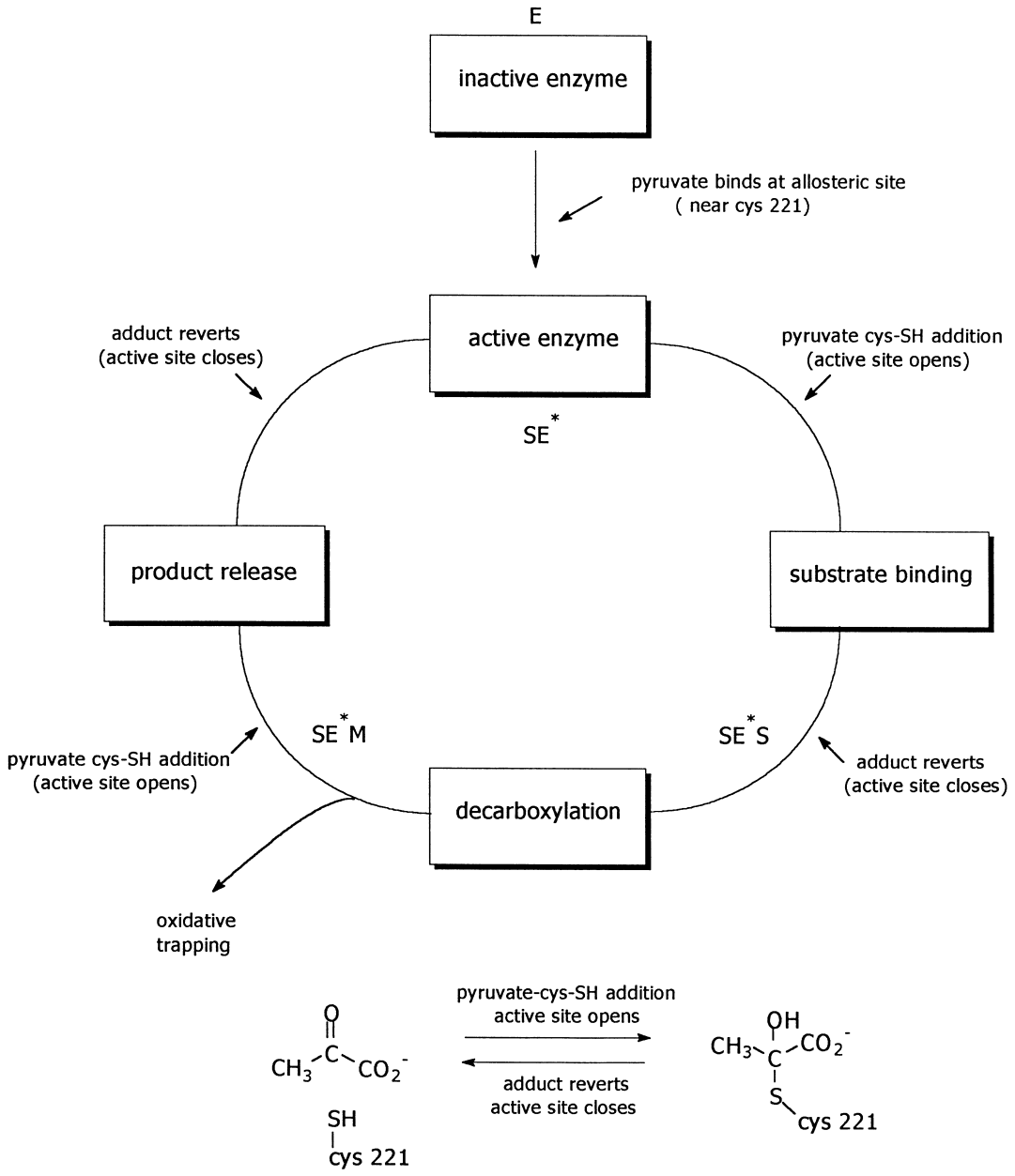

Figure 2. The active-site access model for the hysteretic, allosteric regulation of SCPDC. The slow, hysteretic activation is assumed to involve placement of the 'regulatory pyruvate' at the allosteric site of the free enzyme E, in which active-site access is denied. Binding at the allosteric site is indicated by writing S to the left of E, and completion of the slow activation process by the asterisk in SE*. During the catalytic cycle, addition of the sulfhydryl group of Cys-221 to the keto group of the regulator opens the active site and elimination recloses it. The substrate enters the active site of SE* during an open period to produce SE*S. The active site is presumed closed during decarboxylation, which yields SE*M. Protonation and elimination then gives the product complex and product departs during an open period.

to the active-site magnesium ion (which is also coordinated to the diphosphate unit of ThDP). Thereafter sulfhydryl addition to the keto group moves a loop that denies access to the active site, thus opening it for substrate entry or product departure. The main evidence for this view is that an inverse solvent isotope effect around 2 (i.e., the rate is twofold faster in deuterium oxide than protium oxide) is observed both in substrate binding to the activated enzyme (in every catalytic cycle) and in product release to regenerate the activated enzyme (also in every catalytic cycle). An inverse isotope effect of around twofold is expected for sulfhydryl addition reactions.[17,18] According to this view, the catalytic machinery of SCPDC is fully functional in the inactive enzyme but the enzyme cannot bind substrate or release product.

Paracatalytic intervention in SCPDC action by oxidizing agents

A useful approach to contributing to a discrimination between the two regulatory models is offered by an observation of Christen and his co-workers.[19] They noted that oxidizing agents such as dichlorophenolindophenol (**D**; Fig. 3) can reduce the rate of acetaldehyde formation during the catalytic cycle of

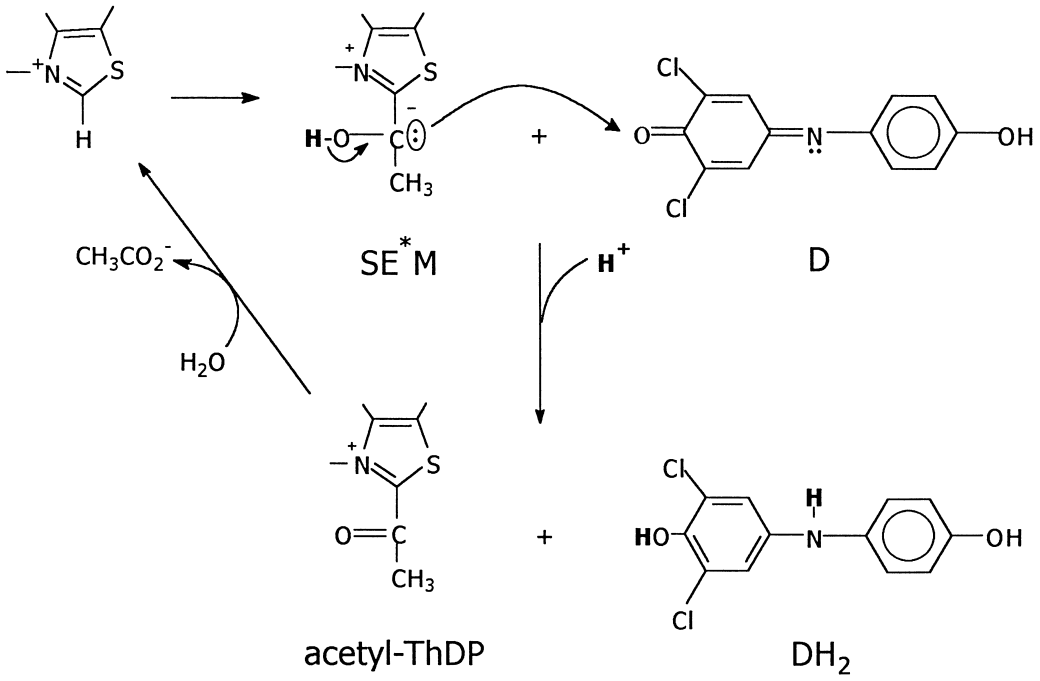

Figure 3. The concept of paracatalytic oxidative diversion during SCPDC action.[19] The oxidant dichlorophenolindophenol (**D**) traps the product of decarboxylation, the 'second carbanion' SE*M. Oxidation leads to acetyl-ThDP which then hydrolyzes to release acetate and complete the catalytic cycle.

SCPDC although the agents have no effect on SCPDC in the absence of pyruvate. Christen[19] termed such reactions *paracatalytic reactions*; it seems very likely that an intermediate in the normal enzymic reaction is being oxidatively trapped and diverted to the formation of alternative products. In the case of **D**, the product was shown to be acetic acid. Thus the likely route of diversion is trapping of the 'second carbanion' that results from decarboxylation, as shown in Figure 3. This converts the intermediate to acetyl-ThDP and alters the mechanism of the product release event. Solvent isotope effects on the unnatural product release event could therefore be informative about the regulation of product release.

We report here the kinetic and isotope-effect characterization of the oxidative diversion reaction of **D** in the action of SCPDC.

Results

The oxidative diversion of the SCPDC-catalyzed decarboxylation of pyruvate by dichlorophenolindophenol (**D**), followed by the spectral change at 520 nm attendant upon reduction of **D**, exhibits roughly hyperbolic saturation (Fig. 4). The concentration of pyruvate in these experiments was 30 mM so that the normal catalytic kinetics were fully saturated. Some inhibition by **D** appears to set in at high concentrations. The velocity v can be described by eq (1), where e_o is the enzyme concentration (molarity of tetrameric SCPDC) and the other constants are defined below.

$$v/e_o = k_{ox}[\mathbf{D}]/\{K_{ox} + [\mathbf{D}](1 + \{K_{inh}/[\mathbf{D}]\})\} \qquad (1)$$

Figure 4 shows specific velocities as a function of the concentration of **D** in both protium and deuterium oxides. The data in each case were fitted to eq (1). The value of the inhibition constant K_{inh} is poorly determined in both cases (339 ± 130 μM in protium oxide, 307 ± 109 μM in deuterium oxide), as is also the case for the similar constant of similar magnitude that is observed in the normal kinetics of SCPDC action. As was done in the treatment of the normal kinetics,[20] a constant value equal to the mean (323 μM) of the fitted values for the two isotopic solvents was therefore used for both data sets. The values of the parameters of eq (1) and the isotope effects resulting from them are shown in Table 1.

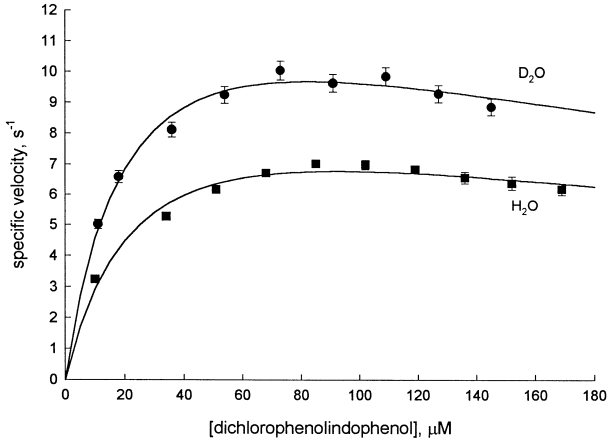

Figure 4. Specific velocity of the oxidative diversion reactions versus oxidant concentration at a saturating concentration of pyruvate. Filled circles refer to reaction in deuterium oxide, filled squares to reaction in protium oxide. The solid lines are plots of eq (1) with the parameters shown in Table 1.

Table 1. Kinetic constants for the SCPDC-catalyzed oxidative diversion of pyruvate by dichlorophenolindophenol in protium and deuterium oxides at 30 °C and pH 6.2 and equivalent

Kinetic constant[a]	H_2O	D_2O	Isotope effects H_2O/D_2O
k_{ox}, s^{-1}	10.7 ± 0.3	14.6 ± 0.3	$1/(1.37 \pm 0.04)$
$10^6 K_{ox}$, M	26.4 ± 2.7	21.6 ± 1.7	1.22 ± 0.16
$10^{-5}\, k_{ox}/K_{ox}$, $M^{-1}\, s^{-1}$	4.0 ± 0.4	6.8 ± 0.6	$1/(1.68 \pm 0.22)$
$10^6 K_{inh}$, M	(323)[b]	(323)[b]	–

[a]Defined by eq (1) of the text.
[b]Fixed at the mean of values for H_2O and D_2O.

Discussion

Kinetic model for the oxidative-diversion reaction

The results in Figure 4 are consistent with several models of the oxidative-diversion reaction, but we would like to give special consideration to the model shown in Figure 5. This model omits the weak inhibition seen at **D** 0.1 mM, which we do not regard as mechanistically significant. This allows the kinetic law of eq (1) to be simplified to that of eq (2), with the parameters defined as shown in eqs (3) and (4) in terms of the microscopic rate constants of Figure 3.

$$\nu/e_o = k_{ox}[\mathbf{D}]/\{K_{ox} + [\mathbf{D}]\} \quad (2)$$

$$k_{ox} = \varphi k_{o3} k_{or}/(\varphi k_{o3} + k_{or}) \quad (3a)$$

where:

$$k_{or} = k_{o5} k_{o7}/(k_{o5} + k_{o7}) \quad (3b)$$

$$\varphi = k_{o5}/(k_{CD} + k_{o5)} \quad (3c)$$

$$k_{ox}/K_{ox} = \{k_{o1} k_{o3}/(k_{o2} + k_{o3})\}\{k_{c5}/(k_{c3} + k_{c5})\}\varphi \quad (4)$$

These rate-constant expressions appear complex but are rational upon examination. The definition of k_{ox} in eq (3a) describes the steady-state first-order rate constant for a sequential process in which decarboxylation in the presence of oxidant **D** (k_{o3} step) is followed by oxidation and product release (rate constant k_{or}, defined in terms of the two rate constants k_{o5} and k_{o7} in eq (3b)). The contribution of the rate constant k_{o3} is modified by the fraction φ, which measures the relative rate of oxidative diversion of SE*MD (see the caption to Fig. 4 for an explanation of the symbols for enzyme forms) against reaction of SE*MD with a proton and elimination to generate acetaldehyde.

The expression for k_{ox}/K_{ox} can also be considered reasonable. The first factor in brackets gives the steady-state second-order rate constant for conversion of SE*S + **D** to $\mathbf{DH}_2$. The second factor in brackets allows for the possible accumulation of SE*M, which would be inhibitory for the oxidative-diversion reaction on this model. We know from studies of the normal catalytic reaction that this factor is about 1/2 in protium oxide and about 1/3 in deuterium oxide.[20] The third factor is then the fraction of oxidative diversion of the 'second carbanion' SE*MD.

The simplest variations on this model, also consistent with the data of Figure 4, involve the addition of **D** to the enzyme at other points in the catalytic cycle. The mechanistic data, now to be presented, appear to us to favor the version of Figure 5 over these alternatives.

Values of the observed rate constants

Table 2 compares the numerical values of the kinetic parameters for oxidative diversion (the regulatory activation of the enzyme having been effected by pyruvate) with related parameters for the normal catalytic reactions of pyruvate (regulatory activation by pyruvate)[20] and of fluoropyruvate (regulatory activation by fluoropyruvate).[21] The fluoropyruvate reaction presents a useful comparison because in the action of SCPDC on fluoropyruvate a fluoride ion is eliminated at the 'second-carbanion' stage to generate acetyl-ThDP in the active site.[22,23] Acetyl-ThDP is also generated in the oxidative diversion reaction. Thus in both cases, product release of acetate ion requires the hydrolysis of acetyl-ThDP.

The most striking result may well be the constant k_{ox}/K_{ox}, with a value of $4 \times 10^5\, M^{-1}\, s^{-1}$. This value is slightly larger than $2 \times 10^5\, M^{-1}\, s^{-1}$, the value of k/B for the normal catalytic reaction of pyruvate.[20] As eq (5) shows, the rate constant k_{ox}/K_{ox} contains the actual rate constant for binding of **D** to SE*S, $\{k_{o1}\, k_{o3}/(k_{o2} + k_{o3})\}$, multiplied by two fractions that are less than or equal to unity. Thus the second order rate constant for binding of the oxidant is still larger than $4 \times 10^5\, M^{-1}\, s^{-1}$. Previous work[20] shows that the factor $\{k_{c5}/(k_{c3} + k_{c5})\}$ is about 1/2 so that the binding rate constant for the oxidant is no smaller than $8 \times 10^5\, M^{-1}\, s^{-1}$. Furthermore, the rate constant for conversion in the normal catalytic cycle[20] for pyruvate of SE* + S to the transition state for decarboxylation is of the order of $10 \times 10^5\, M^{-1}\, s^{-1}$, nearly equal to the rate constant for reaction of SE*S with **D**. Two reasonable conclusions then suggest themselves. They flow from the fact that the rate constant

Table 2. Comparison of kinetic parameters for normal catalysis and for oxidative diversion by dichlorophenolindophenol in the action of SCPDC at 30 °C and pH 6.2 and equivalent

Kinetic constant	Pyruvate[20]	Fluoropyruvate[11]	Dichlorophenol–indophenol
k, s^{-1}	320	44	11
$10^{-5} k/K$, $M^{-1}\, s^{-1}$	2.1	1.1	4.0
$10^6\, K$, M	1510	417	26
^{DOD}k	1.6	1.9	1/(1.4)
$^{DOD}(k/K)$	1/(1.8)	1/(1.3)	1/(1.7)

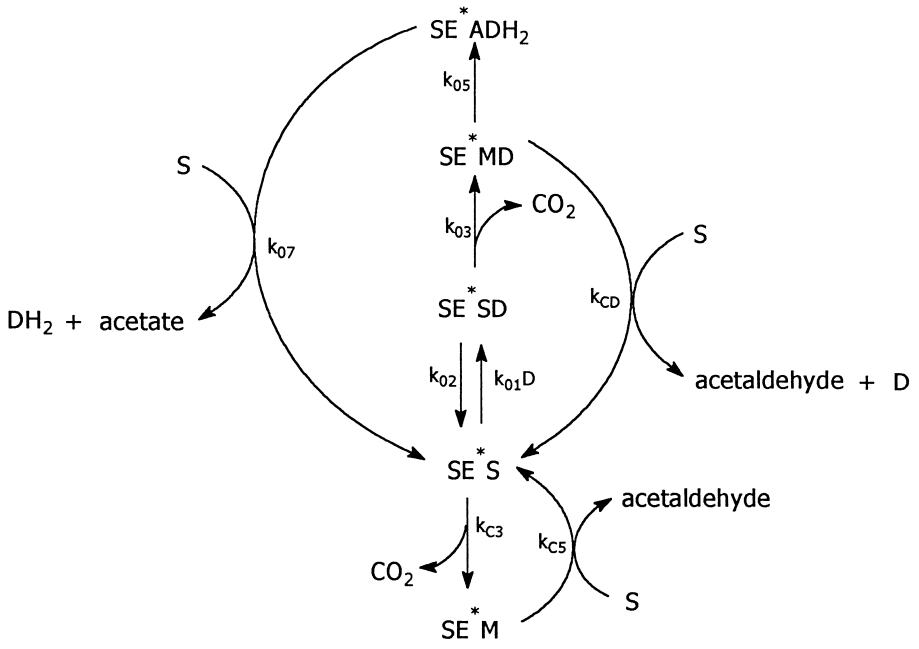

Figure 5. Kinetic model for the oxidative-diversion reaction under conditions of saturating pyruvate. Subscripts c refer to the normal catalytic cycle, and subscripts o to the oxidative diversion. Note that rate constants are in some cases aggregate rate constants for several serial events. The normal catalytic cycle connecting SE*S and SE*M is shown at the bottom (free or unactivated enzyme are not present because of the saturating concentration of S). The oxidative-diversion branch extends upward from SE*S, beginning with entry of **D** into the active site and continuing with decarboxylation in the presence of **D** to generate SE*MD. The mechanism again branches, with protonation of the 'second carbanion' and elimination yielding acetaldehyde while oxidative diversion competitively generates a complex SE*ADH$_2$. This complex contains in the active site **D**H$_2$ and acetyl-ThDP; the latter hydrolyzes and acetate and **D**H$_2$ are released.

k_{ox}/K_{ox} cannot be greater than the rate constant for conversion of SE*S + **D** to the transition state for decarboxylation, the first irreversible step in the reaction sequence. First, unless decarboxylation is somehow *accelerated* by the presence of the oxidant **D** in the active site, the oxidant must be entering the active site rapidly and reversibly and the rate constant k_{ox}/K_{ox} must be that for conversion of SE*S + **D** to the transition state for decarboxylation. Second, the value of φ in eq (4) must be very close to unity. If it were sensibly smaller than unity, the value of $\{k_{o1}k_{o3}/(k_{o2}+k_{o3})\}$ (describing conversion of SE*S + **D** to the transition state for decarboxylation) would become larger than the rate constant for conversion of SE* + S to the transition state for decarboxylation in the normal catalytic cycle. This seems unreasonable, so we conclude that (1) k_{ox}/K_{ox} describes formation of the decarboxylation transition state, and (2) that the product of decarboxylation, SE*M, is efficiently trapped by the oxidant **D** (φ = ca.1).

The picture for k_{ox} is illuminated by these conclusions, which suggest that decarboxylation is not retarded by the presence of **D**, and that trapping by the oxidant is efficient. Thus in eq (3a), we may take φ = 1 and $k_{o3} = 640\,s^{-1}$, the value of the corresponding rate constant in the normal catalytic cycle. Since the overall rate constant k_{ox} is only $11\,s^{-1}$, $k_{or} \ll 640\,s^{-1}$ and $k_{ox} = k_{or}$ and the product-release manifold thus governs the value of the observed rate constant. The observed rate constant must then describe some combination of oxidation, acetyl-ThDP hydrolysis, acetate release, and **D**H$_2$ release, following rapid decarboxylation. The isotope effects suggest a somewhat higher definition for this model.

Isotope effects

The solvent isotope effects are given in Table 2 for the reactions of pyruvate (normal catalytic reaction with allosteric regulation by pyruvate),[20] fluoropyruvate (diversion to acetyl-ThDP and fluoride with allosteric regulation by fluoropyruvate),[21] and the paracatalytic oxidative-diversion reaction (allosteric regulation by pyruvate). The isotope effects on the k/K terms are all inverse (faster in deuterium oxide) by factors of 1.3 to 1.8, while those for the k terms are normal (faster in protium oxide) by factors of 1.6 and 1.9 for pyruvate and fluoropyruvate, respectively, but inverse by a factor of 1.4 for the oxidative-diversion reaction.

The isotope effects for the normal catalytic reaction of pyruvate have been most thoroughly analyzed.[20] The current model we are employing for these isotope effects is this:

- Inverse effect of about 2 on k/B: This effect arises from a rapid, reversible addition of the sulfhydryl group of Cys-221 to pyruvate bound at the allosteric site (Fig. 2). This addition–elimination equilibrium is coupled to a rapid opening–closing of access to the active site. The rate-limiting process is approximately the entry of pyruvate into the active site during an open-access period.
- Normal effect of about 1.5 on k: Proton-inventory analysis suggests that this effect has a complex origin. The value of 1.5 is an average of 1 (no isotope effect) on the decarboxylation reaction and 2 for the product-release reaction, which are equally

rate-limiting. The value of 2 for product release is the product of an inverse isotope effect of 2 and a normal isotope effect of 4. The inverse isotope effect of 2 is exactly similar to that for substrate entry into the active site: we take it to arise from a sulfhydryl addition–elimination equilibrium at the allosteric site coupled to active-site opening and closing, with rate-limiting departure of acetaldehyde from the active site during an open period. Concurrent with the export of acetaldehyde is the import of a proton required in the stoichiometry, and this process generates a normal isotope effect of 4.

A proton-inventory study has also been carried out for the solvent isotope effect of 1.9 (Table 2) seen with fluoropyruvate.[21] In this case, there is *no* contribution of an inverse isotope effect and if such an effect is required for product release in the fluoropyruvate reaction, then product release must be a rapid, non-rate-limiting process. The proton inventory is consistent with two steps that are about equally rate-limiting, one with no solvent isotope effect and the other with a normal solvent isotope effect of about 3.4. The former may be fluoride elimination, the latter either ketonization of the elimination product to generate acetyl-ThDP or hydrolysis of acetyl-ThDP, each of the two contributing processes occurring with a rate constant of about $88\ s^{-1}$. If actual release of acetate occurs with a rate constant similar to that for acetaldehyde release in the normal catalytic cycle ($640\ s^{-1}$), then it is about sevenfold faster than the rate-limiting steps.

In the oxidative-diversion reaction, the inverse isotope effect of around 2 is most easily explained if it is assumed that product release, coupled to sulfhydryl addition at the allosteric site, is now rate-limiting. However, unless some unusual effect is at work, the rate of acetate release cannot be rate-limiting here because its rate constant in the fluoropyruvate reaction is much larger than the observed rate constant of $44\ s^{-1}$, whereas the observed rate constant in the oxidative diversion reaction is only $11\ s^{-1}$. This leaves only the release of $\mathbf{D}H_2$. It does seem likely that this hydrophobic dye molecule may have such a large affinity for the active site that it departs less frequently by about 60-fold than the polar acetate molecule. We therefore suggest that the rate-limiting event for k_{ox} is release of $\mathbf{D}H_2$, coupled to sulfhydryl addition at the allosteric site, which produces the inverse isotope effect.

The isotope effects on k/K are all inverse, in agreement with the view that this constant is determined by events during, or subsequent to, the entry of substrate or oxidizing agent into the active site during an open period in the rapid open–closed cycling driven by reversible addition of Cys-221 to pyruvate at the allosteric site. It should be noted that the true inverse isotope effect for oxidant entry in oxidative diversion may be larger than the measured value of 1.7 for k_{ox}/K_{ox}. The rate constant k_{ox}/K_{ox} is given by $\{k_{o1}\ k_{o3}/(k_{o2}+k_{o3})\}\{k_{c5}/(k_{c3}+k_{c5})\}\varphi$ where, as has been noted, the second factor is about 1/2 in protium oxide and about 1/3 in deuterium oxide and φ is about unity. The inverse isotope effect on $\{k_{o1}\ k_{o3}/(k_{o2}+k_{o3})\}$ which should approximate that for oxidant entry into the active site is therefore $1.7\times1.5=2.6$.

Conclusion

The oxidative-diversion reaction of the SCPDC-pyruvate system by the oxidant dichlorophenolindophenol appears to occur by entry of the oxidant into the active site with a second-order rate constant k_{ox}/K_{ox} and solvent isotope effect very similar to that for the binding of pyruvate to the active site. The solvent isotope effect arises from sulfhydryl addition at the allosteric site, coupled to opening of access to the active site. This process is required for admission of the oxidant to the active site, as well as the normal substrate pyruvate and the abnormal substrate fluoropyruvate.

The first-order rate constant rate k_{ox} reflects a quite slow release of the hydrophobic product of oxidative diversion $\mathbf{D}H_2$, also with an inverse solvent isotope effect arising sulfhydryl addition at the allosteric site, coupled to opening of the active site to permit product departure.

The inverse isotope effects for both oxidant entry into the active site and reduced-product exit from the active site are therefore readily reconciled with the model of allosteric regulation according to which sulfhydryl addition by Cys-221 to the keto group of a pyruvate or other ketone at the allosteric site drives a rapid opening–closing of access to the active site. It is less easy to see how the observations can be reconciled with a model for regulation according to which events at the allosteric site effect reorganization of the catalytic machinery at the active site.

Experimental

Materials. Thiamin diphosphate hydrochloride ('cocarboxylase'), buffers, salts, and NADH were purchased from Sigma, standard solutions, anhydrous citric acid from Fisher, sodium citrate dihydrate and magnesium sulfate (anhydrous) from J. T. Baker. Deuterium oxide was obtained from Aldrich (99.9% D). Sodium pyruvate was obtained from Sigma in 99% pure crystalline form.

Enzyme. Pyruvate decarboxylase (EC 4.1.1.1) from *Saccharomyces cerevisiae* (specific activity 12–14 units/mg) was purchased from Sigma, suspended in a solution of 5% glycerol, 3.2 M ammonium sulfate, 5 mM potassium phosphate, 1 mM magnesium acetate, 0.5 mM EDTA, and 25 FM ThDP, pH 6.5.

2,6-Dichlorophenolindophenol (D). Obtained from Sigma, dissolved in 1 M HCl, and extracted into ether. The sodium salt was re-extracted into 2% sodium bicarbonate solution and precipitated with sodium chloride. The precipitate was washed with sodium chloride solution, dried at 100 °C, and stored in a dessicator.

Solutions in deionized water were stable at 4 °C for two weeks. Concentrations of **D** were determined by spectrophotometric titration at 520 nm (λ_{max} 600 nm) with standard ascorbic acid (prepared by iodate titration). The extinction coefficient of **D** in citrate buffer at pH 6.2 was measured as $(8.28 \pm 0.02) \times 10^3\ M^{-1}\ cm^{-1}$. $\mathbf{D}H_2$ has no measurable absorbance at this wavelength.

Kinetics. The absorbance at 520 nm was followed after injection into a solution containing 30 mM pyruvate, 0.6 U/mL PDC, 0.2 mM ThDP, 10 mM magnesium ion and 0.1 M citrate buffer of an aliquot of the stock solution of **D** such that its concentration was between 10 and 170 μM. Absorbance-time curves were initially linear and the slope was taken to obtain the rate. Rates obtained in this manner were strictly proportional to the enzyme concentration. Specific rates versus [**D**] were then fitted to eq 1 by non-linear least-squares procedures. Reactions in deuterium oxide were carried out at the same buffer ratio as in protium oxide, assuring the corresponding location in the two solvents on the pH(D)-rate profile ('corresponding pL').[17,18]

Background reactions. Each measured rate was corrected for a slow background reaction of **D** with ThDP. No change in absorbance could be detected when **D** was incubated with enzyme, pyruvate, or buffer.

Acknowledgements

We are grateful to Professor Chi-Huey Wong for the opportunity to contribute to this memorial issue. This work was supported by a grant from the National Institute of General Medical Sciences. RLS thanks the Alexander von Humboldt Foundation.

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