Stereochemistry and Function of Oxaloacetate Keto-enol Tautomerase*

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Oxaloacetate keto-enol tautomerase, partially purified from porcine kidney, catalyzes the conversion of enol- to keto-oxaloacetate by a mechanism in which solvent protons end up equally distributed between the two prochiral positions at C3 of keto-oxaloacetate. This conclusion is based upon the observation that when enzyme catalyzed ketonization is conducted in 

$^3$H$_2$O in the presence of excess malate dehydrogenase and NADH, only 50% of the $^3$H in the isolated (2S)-[3,$^3$H]malate is labilized to solvent upon treatment with fumarase. From a stereochemical perspective, this enzyme is unlike phenylpyruvate keto-enol tautomerase that is known to catalyze stereospecific proton transfer between solvent and the pro-R position of keto-substrate. As a result of an attempt to clarify the physiological importance of oxaloacetate tautomerase activity, keto-oxaloacetate was demonstrated to be directly transported across the inner membrane of rat liver mitochondria on the basis of the results of kinetic and isotope-trapping experiments.

Oxaloacetate tautomerase (EC 5.3.2.2) catalyzes one of the most elementary chemical transformations of oxaloacetate (Equation 1 (1)).

\[
\begin{align*}
\text{HO} & \quad \text{C=CHCO}_2^- \\
\text{O} & \quad \text{C} \\
\text{O} & \quad \text{C} \\
\text{O} & \quad \text{C} \\
\end{align*}
\]

\(1\)

Phenylpyruvate keto-enol tautomerase and indolylypyruvate keto-enol tautomerase comprise the remaining known members of this general class of enzymes (2, 3). Although the physiological utility of oxaloacetate tautomerase has not been firmly established, the suggestion that this enzyme plays a crucial role in the metabolism of oxaloacetate is supported by the fact that tautomerase activity is widely distributed among plant, animal, and microbial extracts (1). Indeed, tautomerase activity is found in all compartments of the cell in which the metabolism of oxaloacetate occurs (4).

In order to evaluate the general stereochemical constraints on enzyme catalyzed tautomerization of a-ketoacids, the stereochemistry of the proton transfer reaction catalyzed by oxaloacetate tautomerase was determined for comparison with that of phenylpyruvate tautomerase, known to involve stereospecific exchange of the pro-R proton of keto-substrate with solvent (5). Contrary to expectation, enzymic ketonization of enol-oxaloacetate involves stereorandom incorporation of solvent protons at C3 of keto-oxaloacetate. Thus, oxaloacetate tautomerase must operate on the basis of a highly unusual stereomechanistic principle, provided the physiological role of this enzyme has been correctly assigned.

EXPERIMENTAL PROCEDURES

Materials—The following enzymes and substrates were purchased from Sigma: malate dehydrogenase (porcine heart), fumarase, NADH (grade II) and oxaloacetate (BDH), and NADH (5.2 mM) malate were purchased from New England Nuclear and Amersham-Searle, respectively. The (2S,3R)-[1-14C, 3-3H]malate was prepared by incubation of (S)-[1-14C]malate in $^3$H$_2$O in the presence of fumarase and then purified by anion exchange (Dowex-Cl) and by ion exclusion chromatography (see below). All other reagents were of the highest purity commercially available.

Analytical Methods—High performance liquid chromatography was carried out using a Waters Model 600A solvent delivery system equipped with a U6K sample injection system. Column effluent was monitored at 207 nm with an LKB Ulvacord-8 UV Monitor. Samples for liquid scintillation counting were prepared in Scint-A mixture (Packard) and counted on a Pias Liquid Scintillation Spectrometer. NMR spectra of solutions of oxaloacetic acid in $^2$H-dioxane were taken on an IBM NR-80 instrument. Kinetic measurements were carried out using a Gilford 2400-2 Spectrophotometer. Malate was quantified by the malate dehydrogenase/hydrazine method (6). Transport kinetics of oxaloacetate into rat liver mitochondria were monitored using a Britton Chance Dual, Wavelength Spectrophotometer constructed by Dr. Thomas Marsho (University of Maryland Baltimore County) according to specifications described elsewhere (7).

Purification and Assay of Oxaloacetate Tautomerase—The tautomerase from porcine kidneys was purified initially by the procedure of Annette and Kosicki (1) through the 40–60% (NH$_4$)$_2$SO$_4$ precipitation step to a specific activity of ~3 units/mg. After dissolution of the precipitate in a minimal volume of Tris buffer (5 mM, pH 7) containing EDTA (0.1 mM) and dithioerythritol (0.01 mM), the protein solution was extensively dialyzed against the same buffer and then fractionated on two successive DEAE-cellulose columns equilibrated at different pH, (see Fig. 1). Protein concentrations were determined on the basis of an E$_{280}$ = 2.4 mg/mg-cm.

Tautomerase activity was determined in the direction of ketonization using a coupled enzyme assay in which keto-oxaloacetate is trapped with excess NADH and malate dehydrogenase (1). Solid oxaloacetic acid, evaporated onto a small glass spoon from an ether stock solution (~0.05 μmol of enol-tautomer), was rapidly introduced into a cuvette containing 1 ml of potassium phosphate buffer (10 mM, pH 7.4) containing EDTA (0.1 mM) and dithioerythritol (0.01 mM), the protein solution was extensively dialyzed against the same buffer and then fractionated on two successive DEAE-cellulose columns equilibrated at different pH (see Fig. 1). Protein concentrations were determined on the basis of an E$_{280}$ = 2.4 mg/mg-cm.

Stereochemical Analysis—The strategy for determining the stereochemistry of the tautomerase reaction is depicted in Table I. In order to initiate the stereochemistry experiment, an aliquot of oxaloacetic acid (enriched in enol-tautomer) was introduced into a buffered solution containing tautomerase, excess

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malate dehydrogenase and NADH, as well as $^3$H$_2$O and a trace amount of (S)-[U-14C]malate. After incubation for 30 s (25 °C), the enzymes were removed by ultrafiltration through a PM-10 membrane and the filtrate adsorbed to a Dowex-Cl column (7 × 0.3 cm). The column was first washed with water to remove excess $^3$H$_2$O and then the malate was eluted with aqueous H$_3$SO$_4$ (15 mM). A portion of the pooled malate was further resolved by high performance liquid chromatography using an ion exclusion column (ORH-801 Organic Acids Column, 7/8-inch outer diameter × 30 cm) purchased from Interaction Chemicals. Using aqueous H$_3$SO$_4$ (5 mM) as an eluting solvent, the elution volume of the malate was ~5 ml. The $^3$H/14C ratio for the isolated malate was then determined. The remaining pooled malate from the Dowex-Cl column was brought to dryness with a stream of dry air at 37 °C, dissolved in sodium phosphate buffer (5 mM, pH 7) and incubated with fumarase (23 units) for 30 min. The mixture was then ultrafiltered through a PM-10 membrane and resolved on the ion exclusion column as described above. Fumarase and $^3$H$_2$O co-migrate on the ion exclusion column with an elution volume of ~8.5 ml and, therefore, are well resolved from malate. The $^3$H/14C ratio for the malate peak was determined and compared with that of the malate before treatment with fumarase in order to infer the stereochemistry of the tautomerase reaction.

Preparation of Mitochondria—The mitochondria used in the transport experiments were prepared from the livers of starved Sprague-Dawley rats by a modification of the “high-yield” procedure of Pederson et al. (8), in which 0.25 m sucrose was used in place of 0.22 m mannitol, 0.07 m sucrose. Acceptor control ratios were in the range 0.01–0.02. The mitochondria were loaded with phosphate according to the procedure of Passarella et al. (9). Mitochondrial protein was determined by the Biuret method (10).

RESULTS

Purification and Molecular Weight of Oxaloacetate Tautomerase—In the isolation procedure described originally by Annette and Kosicki (1), oxaloacetate tautomerase was initially purified by heat, acetone, and (NH$_4$)$_2$SO$_4$ fractionation followed by column chromatography using hydroxyapatite prepared by the method of Massey (11). The reported highest specific activity was ~5 units/mg. However, in our hands the column procedure, using commercial hydroxyapatite (Bio-Rad), typically resulted in poor yields of enzyme having ~10-fold lower specific activity than that of the enzyme preparation applied to the column (~5 units/mg).

Subsequently, DEAE-cellulose chromatography was discovered to be an efficient and reproducible method for further purifying the dialyzed enzyme from the (NH$_4$)$_2$SO$_4$ precipitation step. The successive use of two such columns, equilibrated at different pH, resulted in a four-fold increase in specific activity (Fig. 1). The phenylpyruvate tautomerase activity eluted in the pregradient wash of the second DEAE-cellulose column, well separated from the oxaloacetate tautomerase activity. The pooled enzyme from fractions 50–62 of the second column was used in the stereochemistry experiment (specific activity = 11.5 units/mg). Attempts at further increasing the specific activity of the enzyme by gel-filtration chromatography and by affinity chromatography, using Sepharose-4-ethyl-oxaloacetate, were largely unsuccessful due to a marked decrease in the stability of the enzyme as extraneous protein is removed. The inclusion of dithioerythritol (0.01 mM) in all buffers used in the purification procedure marginally increased the stability of the enzyme.

The molecular weight of oxaloacetate tautomerase was estimated to be ~55,500 by gel-filtration chromatography using a Sephadex G-200 column (2 × 49.5 cm) with enzyme from the (NH$_4$)$_2$SO$_4$ fractionation step. The partition coefficient for tautomerase activity ($K_{av} = 0.519$) was compared with those of the following molecular weight standards: malate dehydrogenase (porcine heart) ($K_{av} = 0.439, M_r = 70,000$ (12)), ovalbumin ($K_{av} = 0.575, M_r = 45,000$ (13)), and papain ($K_{av} = 0.826, M_r = 23,000$ (14)).

![Fig. 1. Elution profile of protein and tautomerase activity from two successive DEAE-cellulose columns (1.5 × 11 cm) equilibrated at two different pH values. Panel A shows an elution profile of 939 units of tautomerase from the column equilibrated with Tris buffer (5 mM, pH 6.9), containing EDTA (0.1 mM) and dithioerythritol (0.01 mM), 2 ml/fraction. Approximately 800 units of tautomerase activity eluted between fractions 10 and 20. An additional 26 units of tautomerase was eluted from the column by application of a linear NaCl gradient (0–0.5 m). Fractions 10–20 were pooled, concentrated by ultrafiltration (Millex 100), and reapplied (~175 units) to the same column equilibrated with the same buffer only at pH 7.4 (panel B). Approximately 6 units of tautomerase activity appeared in the pregradient fractions, whereas ~50 units of activity were recovered after initiation of the NaCl gradient.](image-url)

Composition of Oxaloacetic Acid in Dioxane Solution and in the Solid State—Determination of the stereochemistry of enzymic ketonization involved introducing enol-oxaloacetic acid into a coupled enzyme system composed of tautomerase and malate dehydrogenase (Table I). Since enol-oxaloacetic acid is capable of cis-trans isomerism, a means of introducing a single isomer of this tautomer to the coupled enzyme system had to be found in order to avoid ambiguities in the interpretation of the results of the stereochemistry experiment. Solid oxaloacetic acid was initially excluded for this purpose, given the uncertainty over the isomeric composition of the solid (15 and references therein).

A dioxane solution of oxaloacetic acid was finally selected as the source of the enol-tautomer, since a single isomer of this tautomer appears to predominate in dioxane solution on the basis of NMR measurements. In $^1$H-dioxane, 10.06 ppm of oxaloacetic acid exhibits a single, major vinyl proton resonance at 65.83 (Fig. 2). This accords with a previously published spectrum of oxaloacetic acid in the same solvent (15). The presence of significant concentrations of both cis and trans isomers should have been revealed by two well-resolved singlets due to the vinyl protons of each isomer with a separation of roughly 0.3 ppm. This prediction is based upon the difference in the chemical shifts of the vinyl protons due to the cis (65.96) and...
Stereochemistry of Oxaloacetate Tautomerase

TABLE I
Stereochemical analysis of the oxaloacetate tautomerase (OAT) catalyzed ketonization of enol-oxaloacetate (enol-OAA) in H₂O demonstrating stereorandom incorporation of ³H into keto-³H-oxaloacetate

| Experiment | Composition of reaction mixtures* | Specific radioactivity (³H) of malate² |
|------------|---------------------------------|--------------------------------------|
|            | OAT | MDH¹ | [enol-OAA]⁴ | ³H₂O | Before fumarase | After fumarase |
|            | units | units | μmol | (counts/min/μmol) x 10⁻⁴ | (counts/min/μmol) x 10⁻⁴ |
| 1'         | 10.3 | 97.8 | 1.44 ± .09 | 12.46 ± .08 | 6.18 ± .37 | 3.11 ± .18 |
| 2'         | 0    | 48.8 | 1.04 ± .07 | 10.88 ± .01 | 1.48 ± .11 | 0.78 ± .06 |

* Conditions: K₂HPO₄ (10 mM, pH 7.4); NADH (6 mM, pH 7.0); EDTA (0.01 mM); NaCl (9 mM); 1% dioxane; 25 °C; total volume = 1 ml.

¹ Calculated as being equal to (³H/¹⁴C) ([U-¹⁴C]malate)/[enol-OAA], where (³H/¹⁴C) is the ratio of ³H to ¹⁴C counts in the isolated malate.

² MDH, malate dehydrogenase.

³ Introduced from a stock solution of oxaloacetic acid in dioxane prepared 10 min before use.

⁴ Fraction of ³H liberated to solvent from the pro-R position of the isolated (2S)-[³-³H]malate after incubation with fumarase.

⁵ Also contained trace [U-¹⁴C]malate (2.43 ± 0.02) x 10⁶ cpm.

⁶ Also contained trace [U-¹⁴C]malate (2.01 ± 0.1) x 10⁶ cpm.

FIG. 2. The 80-MHz-³H spectrum of a solution of oxaloacetic acid (0.2 m) in d₆-dioxane -1 h after the sample was prepared by dissolution of solid oxaloacetic acid in this solvent. Tetramethylsilane was present as a reference standard. Vinyl protons, due to enol-oxaloacetic acid, appear at 55.93; methylene protons, due to keto-oxaloacetic acid, appear at 53.78. The broad resonance at 58.7 is due to the OH protons of the tautomers. The resonance at 53.5 is due to protiodioxane. The inset shows the relative magnitudes of the vinyl and methylene proton resonances ~15 min after preparation of the sample and, therefore, before equilibrium has been established between the enol- and keto-tautomers.

trans (56.35) isomers of the model compound, [O-alkyl]enol-oxaloacetic acid, calculated on the basis of Pascal’s shielding constants (16). Careful inspection of Fig. 2 reveals a minor resonance at 56.21 that could be attributed to the alternate isomer of the enol-tautomer. However, unlike the resonances at 55.93 and 53.79, the magnitude of this resonance was undiminished by the addition of an aliquot of ³H₂O to the NMR sample, indicating that this resonance is not due to an exchangeable proton. Finally, although shielding constant calculations can be subject to substantial error, the above analysis is nevertheless indicative of the predominance of a single isomer of enol-oxaloacetic acid in dioxane solution. No claim is made as to which isomer accounts for the resonance at 55.93.

The discovery that the ketonization of enol-oxaloacetic acid is a slow process in d₆-dioxane solution provided a method for establishing that solid oxaloacetic acid is composed of the same isomer of the enol-tautomer that predominates in d₆-dioxane solution (Fig. 2, inset). The time-dependent decrease in the integrated intensity of the vinyl proton resonance after dissolution of solid oxaloacetic acid in d₆-dioxane is a first order process, kobs = 0.038 min⁻¹ (52 °C). Thus, the fraction of enol-tautomer present at the time of dissolution of solid oxaloacetic acid in d₆-dioxane (A½) could be calculated from ln ([A½ - Aₗ]/[A₀ - Aₗ]) = -kobs·t where A₀ = percent enol-tautomer at any time t, and Aₗ = percent enol-tautomer at t = inf, corresponding to 68% enol-tautomer and 32% keto-tautomer. Thus, A½ was calculated to be 190 ± 5%. Consistent with this conclusion is the fact that the calculated activity of oxaloacetate tautomerase is independent of whether the enzymic assay is initiated with solid oxaloacetic acid or with a solution of oxaloacetic acid in dioxane.

Nonenzymic Ketonization of Enol-oxaloacetic acid—In order to calculate the fraction of enol-oxaloacetic acid actually converted to keto-oxaloacetic acid by the tautomerase in the stereochemistry experiment, the rate constant for nonenzymic ketonization of enol-oxaloacetic acid had to be determined under the buffer conditions described in Table I. This was done by diluting an aliquot of oxaloacetic acid in dioxane solution (0.02 ml, 3.34 μmol), prepared 1 h before use, into buffer (2 ml) containing excess malate dehydrogenase (12 units) and NADH (6 mM) in a 1-cm cuvette and then following the time-dependent loss in optical density at 390 nm due to oxidation of the NADH. The initial rapid loss in optical density was due to rapid reduction of the keto-tautomer, the direct substrate for malate dehydrogenase. This represented 29.3 ± 5.7% of the total oxaloacetate present, in fair agreement with the results of the NMR experiment. The slower first order loss in optical density that followed was attributed to rate-limiting ketonization of enol-oxaloacetic acid, k = 0.40 ± 0.01 min⁻¹.

Stereochemistry of the Oxaloacetate Tautomerase Reaction—The stereochemistry of solvent proton incorporation into enol-oxaloacetic acid, as catalyzed by the tautomerase in ³H₂O, was determined using a coupled enzyme reaction in
which the keto-[3-3H]oxaloacetate was trapped as (2S)-[3-3H]malate by excess malate dehydrogenase and NADH (Table 1). The coupled enzyme reaction was initiated using a stock solution of oxaloacetic acid in dioxane solution that had been prepared shortly (10 min) before use, in order to ensure near maximal enrichment of the dioxane solution in the enol-tautomer (96%). The distribution of 3H between the pro-R and pro-S positions of the isolated (2S)-[3-3H]malate, and hence of the keto-[3-3H]oxaloacetate, was then determined on the basis of the fraction of 3H liberated from (2S)-[3-3H] malate to solvent upon incubation with fumarase. Fumarase catalyzes the reversible anti-elimination of water from (S)-malate using the pro-R proton (17).

The coupled enzyme system was designed to minimize nonenzymic incorporation of 3H into the keto-[3-3H]oxaloacetate by using excess tautomerase and malate dehydrogenase (Equation 2).

Greater than 95% of the enol-tautomer is calculated to undergo enzymic ketonization, given the initial concentration of enol-tautomer (1.44 mM). Nonenzymic conversion of keto-tautomer back to enol-tautomer is insignificant in the presence of 98 units of malate dehydrogenase, given the small rate constant for nonenzymic enolization calculated on the basis of $K_{enol} \approx [ket0-oxaloacetate]/[enol-oxaloacetate] = 10$ (18, 19).

A control experiment established that once the (2S)-[3-3H] malate is formed in the reaction mixture, the excess malate dehydrogenase will not catalyze further exchange with solvent by reversal of the malate dehydrogenase reaction, followed by nonenzymic tautomerization of keto-oxaloacetate, over the time course of the stereochemistry experiment. Independently synthesized (2S,3R)-[U-14C, 3-3H]malate (0.4 mM; $\text{[^3H]_[^14C]} = 3.90$) was incubated with malate dehydrogenase (48 units) for 30 min in the presence of NAD$^+$ (0.8 mM) and NADH (2.5 mM) in unlabeled water under the buffer conditions described in Table I. After the normal stereochemical workup, the $\text{[^3H]_[^14C]}$ ratio of the isolated (S)-malate was essentially unchanged (3.91). Fumarase treatment of this malate resulted in complete labilization of $\text{[^3H]}$ to solvent, demonstrating that the stereochemical workup does not result in racemization of the malate at C3.

In the experiments described in Table I, the inclusion of a trace amount of (S)-[U-14C]malate in the reaction mixtures allowed an accurate final assessment of the loss of $\text{[^3H]}$ from the isolated (2S)-[3-3H]malate upon incubation with fumarase, on the basis of the decrease in the $\text{[^3H]_[^14C]}$ ratio. The data of Table I demonstrate that both enzymic and nonenzymic ketonization of enol-oxaloacetate in H$_2$O results in the same (R,S)-keto-[3-3H]oxaloacetate.

**Permeability of Liver Mitochondria to Keto-oxaloacetate**

The unusual outcome of the stereochemistry experiment prompted an attempt to clarify the physiological role of the tautomerase in the cell. Heretofore, the significance of this enzyme activity has been unclear, since all of the oxaloacetate-dependent enzymes so far examined use keto-oxaloacetate, the thermodynamically predominant tautomer (80–90%) under physiological conditions (18, 19). Thus, the conditions under which ketonization of the minor enol-tautomer would become physiologically important is uncertain. However, another oxaloacetate-dependent process whose tautomeric specificity has not been examined is the direct transport of oxaloacetate across the inner mitochondrial membrane. This process may be metabolically important under certain physiological conditions (20). Thus, if the translocator protein(s) involved in transport were specific for the enol-tautomer, the need for a tautomerase might be comprehensible, given that the keto-tautomer is the substrate for or the product of both intra- and extramitochondrial oxaloacetate-dependent enzymes.

That oxaloacetate is directly transported across the inner membrane of rat liver mitochondria in vitro was first suggested by the observation that exposure of mitochondria to exogenous oxaloacetate results in a rapid loss of absorbancy due to oxidation of intramitochondrial NAD(P)H, a process catalyzed by intramitochondrial malate dehydrogenase (21). Transport appears to limit the rate of oxidation, since this rate is greater in disrupted mitochondria. Subsequent in vitro studies demonstrated that transport is a saturable process obeying Michaelis-Menten kinetics (9, 21, 22). Transport may involve the so called “dicarboxylate” and/or “a-ketoglutarate” translocator systems (22, 23).

In order to test whether enol-oxaloacetate is the exclusive substrate for transport, mitochondria were exposed alternately to keto- and enol-enriched samples of oxaloacetate and the comparative rates of oxidation of intramitochondrial NAD(P)H followed by dual-wavelength spectrophotometry (Fig. 3).

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1. The abbreviations used are: EGTA, ethylenebis(oxyethyl)-enlenitrilo)tetraacetic acid; HEPES, 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid.
The observation that the initial rate of transport is greater using keto-enriched samples of oxaloacetate shows that the enol-tautomer is not the exclusive substrate for transport, according to the following scheme.

\[
\begin{align*}
\text{Enol-oxaloacetate} & \quad \text{Keto-oxaloacetate} \\
\text{Membrane} & \quad \text{malate dehydrogenase} \\
\text{NADH} & \quad \text{NAD}^+ \\
\text{Sp(0.090, 0.17)x10^5 cmp/μmole} & \quad \text{Sp(1.7910, 0.34)x10^5 cmp/μmole} \\
\text{enol-}[3-3H]oxaloacetate} & \quad \text{keto-}[3-3H]oxaloacetate \\
\text{Sp(Y)=1.7270, 0.22x10^5 cmp/μmole} & \quad \text{Sp(Y)=1.7270, 0.22x10^5 cmp/μmole} \\
\text{Keto-}[3-3H]oxaloacetate} & \quad \text{enol-}[3-3H]oxaloacetate \\
\text{Sp(X)=1.7910, 0.34x10^5 cmp/μmole} & \quad \text{Sp(X)=1.7910, 0.34x10^5 cmp/μmole} \\
\end{align*}
\]

Thus, the keto-tautomer is either the exclusive substrate or a cosubstrate for transport. The observed biphasic kinetics using enol-enriched samples of oxaloacetate is consistent with an increasing rate of transport as the extramitochondrial enol-

**DISCUSSION**

The central observation of this work is that oxaloacetate tautomerase catalyzes the ketonization of a single isomer of enol-oxaloacetate in H_2O to give (R,S)-ketol-[3-3H]oxaloacetate. 

**Stereospecificity of Proton Transfer**—Stereospecific proton transfer is the general rule among enzyme-catalyzed reactions, provided one excludes those enzymes for which an enol intermediate dissociates from the active site during catalysis (24). Nevertheless, two general explanations can be envisioned for the apparent lack of stereospecificity in the proton transfer reaction catalyzed by oxaloacetate tautomerase.

The first of these is based upon the failure to purify the enzyme to homogeneity. Conceivably, the preparation of oxaloacetate tautomerase used in the stereochemistry experiment contained two tautomerase species that catalyze ketonization with similar efficiencies but opposite stereoisomers. This would be an unprecedented explanation in that, to our knowledge, there are no other examples of two enzymes from the same biological source that catalyze the same reaction with opposite stereoisomers. Equally unprecedented is the possibility that a single tautomerase species contains two active sites that catalyze the same reaction with equal efficiencies but with opposite stereoisomers.

A second and seemingly more likely possibility is that enol-oxaloacetate binds to the active site in such a way that both diastereotopic faces of the enol-tautomer are accessible to proton addition by the enzyme (two-base mechanism) or by solvent. A variation of this hypothesis would be obtained if there are different binding modes between substrate and enzyme so that the two diastereotopic faces of bound enol-oxaloacetate alternatively undergo unidirectional protonation by the enzyme (single-base mechanism) or by solvent.

**Relationship to Phenylpyruvate Keto-enol Tautomerase**—Related to the question of mechanism is the more fundamental problem of why oxaloacetate tautomerase catalyzes a non-stereospecific proton transfer when the vast majority of enzymes that catalyze stereochemically cryptic reactions do so stereospecifically (24). The generality of stereospecific catalysis suggests that enzymes evolve partly in response to the need to achieve a high state of catalytic efficiency requiring a well-defined topological relationship between bound substrate and the catalytic residues within the active site. Phenylpyruvate tautomerase, an enzyme of the same reaction type as oxaloacetate tautomerase, conforms to this generality in that
Physiological pH are composed of roughly 80-90% keto-tautomerase catalytic mechanism must be tempered by the possibility that least in the case of the tautomerase, speculation about the boxylase activity of pyruvate kinase may be an example of an which may serve as the direct substrate for the thyroidal contrast, the biological function of phenylpyruvate tautomerase is another example of a chemical process with minimal catalytic requirements (29-32). Indeed, the oxaloacetate decarboxylation reaction has recently been reported to undergo nonstereospecific proton transfer in a fashion analogous to that of a nonenzymic catalyst. Certainly, the requirements for catalysis are minimal in that acids, bases, and metal ions all serve as efficient nonenzymic catalysts of tautomerization (26-28). The decarboxylation of oxaloacetate is also an example of a chemical process with minimal catalytic requirements (29-32). Indeed, the oxaloacetate decarboxylate activity of pyruvate kinase may be an example of an aberrant property of an active site that has evolved for some other purpose (33, 34). The same possibility must be carefully considered in the case of the tautomerase, although this enzyme is known to be distinct from the common oxaloacetate-dependent enzymes citrate synthetase, glutamate-oxaloacetate transaminase, malate dehydrogenase, and fumarase (1). In addition, commercial rabbit muscle pyruvate kinase (Sigma) does not exhibit detectable oxaloacetate tautomerase activity.

In a related vein, the physiological usefulness of tautomerase activity is unclear. Aqueous solutions of oxaloacetate near physiological pH are composed of roughly 80-90% keto-tautomers and approximately equal concentrations of enol-tautomers (18, 19). However, all of the oxaloacetate-dependent biological processes so far examined use the predominant keto-tautomers as substrate or produce it as product. These processes include seven different enzymic reactions (1, 35). In addition, the direct transport of oxaloacetate across the inner membrane of rat liver mitochondria involves the keto-tautomers (Fig. 3, Chart I). Since enol-oxaloacetate has yet to be clearly identified as a biological substrate, the conditions under which tautomerization would influence the metabolism of oxaloacetate are unclear. In contrast, the biological function of phenylpyruvate tautomerase is less problematical, since the enzyme can use as substrate 4-hydroxy-3,5-diidoophenylpyruvate, the enol-tautomers of which may serve as the direct substrate for the thyroidal peroxidase in the biosynthetic pathway leading to thyroxine (36).

**Solvent Tritium Isotope Effects**—Even if tautomerization is an aberrant catalytic process, some degree of stereoselectivity during proton transfer would be expected by virtue of the fact that catalysis takes place on the asymmetric surface of a protein. In apparent contradiction to this expectation is the observation that enzyme-catalyzed ketonization of enol-oxaloacetate gives rise to racemic keto-[3-3H]oxaloacetate. However, this observation does not prove that both diastereotopic faces of the bound enol-tautomeres are equally accessible to protonation, since the stereochemistry may be under thermodynamic rather than under kinetic control.

This possibility is suggested from the comparative magnitudes of the primary solvent deuterium isotope effects for enzymatic versus nonenzymic ketonization of enol-oxaloacetate, calculated from the specific radioactivities of the isolated (2S)-[3-3H]malate with those of the D2O in which the reactions were conducted (Table I). For the nonenzymic case, proton transfer is at least partially rate-determining, kH/kD = (10.88 ± 0.61) × 104/(1.48 ± 0.11) × 104 × 2 = 3.68 ± 0.27. The factor of two in the numerator of this equation accounts for the fact that ketonization involves the incorporation of a single solvent proton, whereas the specific radioactivity of the D2O is calculated on the basis of the two exchangeable protons. For the enzymatic case, the isotope effect is near unity when calculated on the basis of the assumption that only one proton is incorporated from solvent: kH/kD = (12.46 ± 0.08) × 104/(6.18 ± 0.36) × 104 × 2 = 1.01 ± 0.06. This suggests that some other step besides proton transfer is rate-determining, possibly dissociation of keto-oxaloacetate from the protein. Thus, the formation of racemic keto-[3-3H]oxaloacetate by the tautomerase would be consistent with rapid (although possibly unequal) rates of equilibrium protonation of bound substrate, but rather is due to a fortuitous arrangement of amino acid residues that catalyze protonation, since the stereochemistry may be under kinetic control.

Conclusions—The proton transfer reaction catalyzed by oxaloacetate tautomerase is not stereospecific, in contrast to that catalyzed by phenylpyruvate tautomerase. The biological usefulness of oxaloacetate tautomerase activity is unclear. If indeed the tautomerase protein has evolved to specifically bind oxaloacetate, the biological significance of this interaction may be something other than to catalyze the tautomerization of oxaloacetate.

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**REFERENCES**

1. Annett, R. G., and Kosicki, G. W. (1969) J. Biol. Chem. 244, 2059-2067
2. Knox, W. E., and Fitt, B. M. (1957) J. Biol. Chem. 225, 675-688
3. Spencer, R. P., and Knox, W. E. (1962) Arch. Biochem. Biophys. 96, 115-124
4. Weesenberg, J. C., Chandhari, A., and Annette, R. G. (1976) Can. J. Biochem. 54, 233-237
5. Retey, J., Bartl, K., Ripp, E., and Hull, W. E. (1977) Eur. J. Biochem. 72, 251-257
6. Hoborh, H.-J. (1971) in Methods of Enzymatic Analysis (Bergmeyer, H.-U., ed) Verlag Chemie/Academic Press, New York
7. Chance, B. (1951) Rev. Sci. Instrum. 22, 634-638
8. Pederson, P. L., Greenwald, J. W., Reynafarje, B., Hullihen, J., Decker, G. L., Joper, J. W., and Bustamente, E. (1978) in Methods in Cell Biology (Bersch, D. M., ed) Vol. 20, pp. 411-481, Academic Press, New York
9. Passarella, S., Palmieri, F., and Quagliariello, E. (1977) Arch. Biochem. Biophys. 180, 160-168
10. Cooper, T. (1977) The Tools of Biochemistry, p. 52, John Wiley & Sons, New York
11. Massey, V. (1960) Biochem. Biophys. Acta 27, 130-134
12. Thorne, C. J. R., and Kaplan, N. (1963) J. Biol. Chem. 238, 1861-1868
13. Castallino, F. J., and Barker, R. (1968) Biochemistry 7, 2207-2217
14. Weber, K., and Oasborn, M. (1968) J. Biol. Chem. 244, 4406-4412
15. Hess, J. L., and Reed, R. E. (1972) Arch. Biochem. Biophys. 153, 226-232
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16. Pascual, C., Meier, J., and Simon, W. (1966) Helv. Chim. Acta 49, 164–168
17. Gawron, O., and Fondu, T. P. (1959) J. Am. Chem. Soc. 81, 6333–6334
18. Kokesh, F. C. (1976) J. Org. Chem. 41, 3593–3599
19. Emily, M., and Leussing, D. L. (1981) J. Am. Chem. Soc. 103, 628–634
20. Illingsworth, J. A., Ford, W. C. L., Kobayashi, K., and Williamson, J. R. (1975) in Recent Advances in Studies on Cardiac Structure and Metabolism. The Cardiac Sarcoplasm (Roy, P.-E., and Harris, P., eds) Vol. 8, pp. 271–290, University Park Press, Baltimore
21. Haslam, J. M., and Krebs, H. A. (1968) Biochem. J. 107, 659–667
22. Girpel, J. A., DeHaan, E. J., and Tager, J. M. (1973) Biochim. Biophys. Acta 292, 582–591
23. Passarella, S., Palmieri, F., and Quagliariello, E. (1977) in Bioenergetics of Membranes (Packer, L., Papageorgiou, G. C., and Trebst, A., eds) pp. 425–434, Elsevier/North-Holland Biomedical Press, Amsterdam
24. Hanson, K. R., and Rose, I. A. (1975) Acc. Chem. Res. 8, 1–10
25. Rozzell, J. D., Jr., and Benner, S. A. (1984) J. Am. Chem. Soc. 106, 4937–4941
26. Raghavan, N. V., and Leussing, D. L. (1976) J. Am. Chem. Soc. 88, 723–730
27. Covey, W. D., and Leussing, D. L. (1974) J. Am. Chem. Soc. 96, 3860–3866
28. Gelles, E., and Hay, R. W. (1958) J. Chem. Soc. (Lond.), 3673–3683
29. Steinberger, R., and Westheimer, F. H. (1951) J. Am. Chem. Soc. 73, 429–435
30. Kosicki, G. W., Lipovac, S. N., and Annette, R. G. (1964) Can. J. Chem. 42, 2806–2810
31. Kosicki, G. W., and Lipovac, S. N. (1964) Can. J. Chem. 42, 403–415
32. Tsai, C. S., Lin, Y. T., and Sharkawi, E. E. (1972) J. Org. Chem. 37, 85–87
33. Creighton, D. J., and Rose, I. A. (1976) J. Biol. Chem. 251, 61–68
34. Creighton, D. J., and Rose, I. A. (1976) J. Biol. Chem. 251, 69–72
35. Dolin, M. I. (1968) J. Biol. Chem. 243, 3916–3923
36. Blasi, F., Pragomele, R., and Covelli, I. (1969) Endocrinology 85, 542–551