Mechanism of Yeast Cytochrome b_2 Action. II. Steady-State Kinetics of Oxalate Inhibition†

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ABSTRACT: From a careful steady-state kinetic study it is shown that the inhibition of L-lactate oxidation by cytochrome b_2 with ferricyanide as acceptor is of the mixed competitive-noncompetitive type, indicating the formation of an active ternary complex between enzyme, substrate, and inhibitor. With a large excess of acceptor, the simplest formal mechanism consistent with all available data is: E + L $\rightleftharpoons$ EL; E + S $\rightleftharpoons$ ES $\rightarrow$ EP $\rightarrow$ E + P; ES + L $\rightleftharpoons$ ESL $\rightarrow$ EPL $\rightarrow$ EL + P, where L is oxalate, S is L-lactate, P is pyruvate, and E is enzyme. The inhibition kinetics together with the rate constants for oxalate binding to free enzyme (Thusius, D., Blazy, B., and Baudras, A. (1976), *Biochemistry,* preceding paper in this issue) and recent steady-state experiments on L-lactate deuterated at C-2 (Lederer, F. (1974), *Eur. J. Biochem. 46,* 393) lead to estimates of some of the elementary rate parameters in the above scheme. As in the case of oxalate (see Thusius et al. reference above), the association rate constant for substrate binding ($1.1 \times 10^5\ M^{-1}\ sec^{-1}$) is much smaller than a diffusion-controlled value. Our results also imply that dissociation of complex EP to free enzyme and pyruvate is partially rate limiting for the overall reaction.

Cytochrome b_2 efficiently catalyzes the oxidation of L-lactate to pyruvate in the presence of ferricyanide:

$$2Fe(CN)_6^{3-} + \text{L-lactate} \rightarrow 2Fe(CN)_6^{4-} + \text{pyruvate} + 2H^+$$

Investigations aimed at elucidating the mechanism of the above reaction were cited in the introductory statement of the preceding paper (Thusius et al., 1976). The present contribution is concerned with the inhibition of L-lactate oxidation by oxalate. Due to the fortunate circumstance that inhibition in this case is of the mixed competitive-noncompetitive type, some of the elementary rate constants for a minimal kinetic mechanism could be estimated.

Materials and Methods

The enzyme preparation and determination of lactate concentration are described in paper I (Thusius et al., 1976). Only freshly prepared enzyme was used in the steady-state kinetic measurements.

Experimental Results

It is known that over a wide range of ferricyanide and L-lactate concentrations the initial, "steady-state" rates of ferrocyanide production obey a simple Michaelis-Menten rate law (Morton et al., 1961; Hinkson and Mahler, 1963a). In the present work we have investigated the inhibition of this reaction by oxalate. The parameters V_{max}^{app} and K_m^{app} in the Michaelis equation:

$$v = V_{max}^{app}/(1 + K_m^{app}/C_S^0) \quad (1)$$

were evaluated numerically by the nonlinear least-squares procedure of Cleland (1967). At a given inhibitor concentration the initial rate v (where v is the rate of lactate disappearance in $M\ sec^{-1}$) was determined at seven different lactate concentrations between $0.2K_m^{app}$ and $5K_m^{app}$. We chose to work at high ferricyanide concentration (1.5 mM) where K_m^{app} and V_{max}^{app} are independent of acceptor concentration (Table I). In Figure 1 we summarize our results in the form of Eadie plots. It follows from eq 1 that:

$$v = V_{max}^{app} - K_m^{app}(v/C_S^0) \quad (2)$$

Therefore the slopes and y-axis intercepts in Figure 1 equal $-K_m^{app}$ and V_{max}^{app}, respectively. This type of representation suffers less from data dispersion at low substrate concentrations than "double-reciprocal" Lineweaver-Burk plots. The excellent agreement between the data points and the theoretical lines calculated from the parameters of the numerical fitting to eq 1 demonstrates that the enzymatic reaction obeys Michaelis-Menten kinetics at all oxalate concentrations studied.

In purely competitive inhibition (mutually exclusive binding of substrate and inhibitor to the same site; no ternary complex formation between enzyme, inhibitor, and substrate), the slopes of Eadie plots increase with inhibitor concentration while the y-axis intercepts remain constant. In purely noncompetitive inhibition (substrate and inhibitor binding to different, independent sites; no hindrance to ternary complex formation), the slopes remain constant and the intercepts decrease with inhibitor concentration. It is clear from Figure 1 that oxalate exhibits mixed behavior in which both slopes and intercepts are ligand dependent.[1] The most straightforward interpretation is that a ternary complex can form between the Michaelis complex and oxalate. A simple formal mechanism which accounts for the results of Figure 1 is given in Scheme I. It is reasonable to assume that oxalate and L-lactate possess the same number of cytochrome b_2 binding sites (Capeillère, 1974). The kinetic be-

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[1] Conceivably the kinetic behavior of Figure 1 could arise from ionic effects due to the partial replacement of phosphate by oxalate. This seems unlikely in view of the fact that steady-state parameters measured in Tris-Cl (μ = 0.5 M) were essentially identical with those determined in phosphate at the same ionic strength.

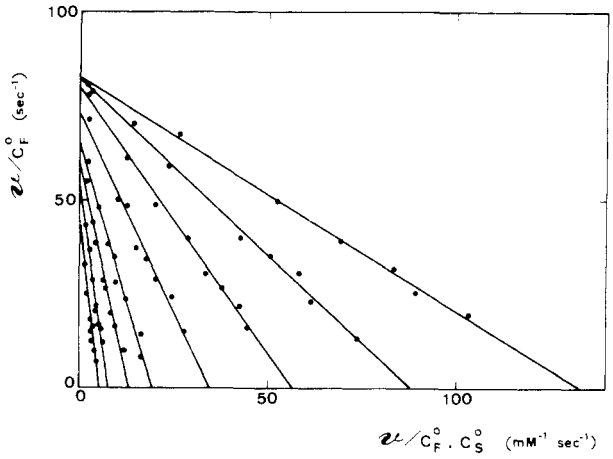

FIGURE 1: Inhibition of cytochrome b_2 by oxalate. Conditions are: 0.5 M ionic strength; phosphate buffer; 1 mM EDTA; pH 7.2; 20°C; acceptor, 1.5 mM potassium ferricyanide. The straight lines from right to left correspond to the following oxalate concentrations: 0, 0.48, 1.14, 2.83, 5.70, 9.30, 14.40, and 22.40 mM. C_F^0 denotes total concentration of catalytic sites. The lines were calculated using eq 1.

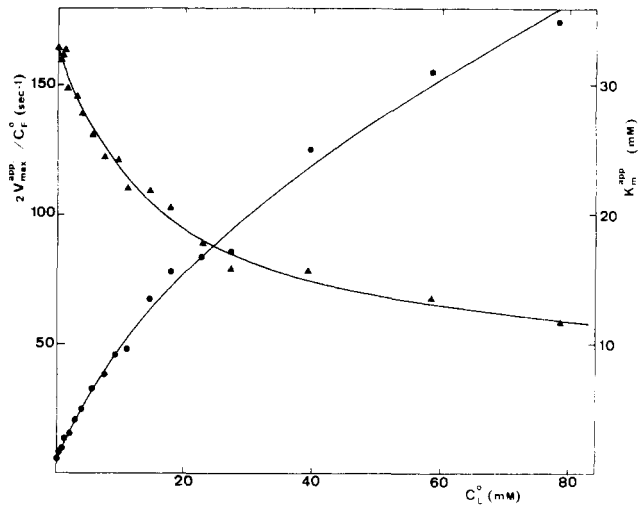

FIGURE 2: K_m^{app} (●) and V_{max}^{app} (▲) as a function of oxalate concentration. The two curves are theoretical fits calculated with the empirical parameters $a, \ldots, f$ given in the text.

Table I: Effect of Ferricyanide on Steady-State Kinetic Parameters in the Presence and Absence of Oxalate.[a]

Ferricyanide (mM)	$C_L^0 = 0$ K_m (mM)	$C_L^0 = 0$ $2V_{max}/C_F^0$ (sec^{-1})	$C_L^0 = 22.4$ mM K_m^{app} (mM)	$C_L^0 = 22.4$ mM $2V_{max}^{app}/C_F^0$ (sec^{-1})
1.50	1.20	165	16.5	90
0.83	1.20	164	16.6	91
0.28	1.02	159	14.0	85

[a] Conditions: 0.2 M phosphate, 1 mM EDTA, pH 7.20, 20°C. C_F^0 denotes the total concentration of substrate binding sites (calculated assuming 1 site per subunit).

Scheme I

$$F + L \underset{k_d}{\overset{k_a}{\rightleftharpoons}} FL$$

$$F + S \underset{k_{-1}}{\overset{k_1}{\rightleftharpoons}} FS \xrightarrow{k_2} F + P$$

$$FS + L \underset{k'_{-1}}{\overset{k'_1}{\rightleftharpoons}} FLS \xrightarrow{k'_2} FL + P$$

havior implies that the sites for substrate and inhibitor partially overlap. To simplify the representation of the multistep mechanism we define a locus of both sites as F. The rate constants of Scheme I are then intrinsic parameters. As discussed in paper I (Thusius et al., 1976), the elementary rate constants are related to intrinsic values by statistical factors. Since at the acceptor concentration used here elementary reactions involving $Fe(CN)_6^{3-}$ are not rate limiting, it is not necessary to include these processes in the above scheme.

Assuming all enzyme species exist in steady states, the following expression may be derived for V_{max}^{app} and K_m^{app} corresponding to Scheme I:

$$V_{max}^{app}/C_F^0 = (a + bC_L^0)/(1 + cC_L^0) \quad (3)$$

$$K_m^{app} = [(1 + dC_L^0)(e + fC_L^0)]/(1 + cC_L^0) \quad (4)$$

where $a = k_2$, $b = k_2'/K_m'$, $c = [1 + (k_2'/k_d)]/K_m'$, $d = 1/K$, $e = K_m$, and $f = k_2'/(k_1K_m')$. The Michaelis constants for the two catalytic paths leading to pyruvate production are given in eq. 5. The constants V_{max}^{app}/C_F^0 and K_m^{app} are together functions of six independent empirical parameters, which are in turn related to the specific rate constants of the microscopic mechanism.

$$K_m = (k_{-1} + k_2)/k_1$$
$$K_m' = (k_{-1}' + k_2')/k_1' \quad (5)$$

Examination of eq 3 reveals that V_{max}^{app}/C_F^0 will decrease monotonically with inhibitor concentration until reaching the plateau value $V_{max}^{app} \rightarrow b/c$. The quantity K_m^{app} increases nonlinearly at intermediate concentrations and linearly at high concentrations, where $K_m^{app} \rightarrow (df/c)(C_L^0)$. The profiles of Figure 2 are qualitatively consistent with this behavior. Although rate data at yet higher oxalate concentrations would provide a more stringent test, this would require working at ionic strengths where comparisons with the present results are not necessarily valid.

An additional series of steady-state kinetic experiments was carried out with enzyme from a second preparation. By repeating some of the runs of Figure 1 it could be shown that up to at least 27 mM oxalate the V_{max}^{app}/C_F^0 and K_m^{app} values differed by no more than 5% from those found in the original study.

The steady-state results were fit to mechanism I with a nonlinear least-squares analysis of V_{max}^{app}/C_F^0 and K_m^{app} as a function of oxalate concentration. In the case of V_{max}^{app}, eq 3 was rearranged to a form linear in one parameter (a) and nonlinear in two parameters (c and b/a):

$$V_{max}^{app}/C_F^0 = a\,\frac{1 + (b/a)C_L^0}{1 + cC_L^0} \quad (6)$$

The data points of Figure 2 gave $a = 81$ (± 1) sec^{-1}, $b/a = 0.014$ (± 0.003) mM, and $c = 0.060$ (± 0.007) mM^{-1}. Therefore we may also conclude that $b = 1.1$ (± 0.2) mM^{-1} sec^{-1}.

The expression for K_m^{app} may be arranged to a form analogous to that of eq 6:

$$K_m^{app}/(1 + dC_L^0) = e\,\frac{1 + (f/e)C_L^0}{1 + cC_L^0} \quad (7)$$

The left-hand side of the above relation was calculated from the experimental K_m^{app} and the titration value of $d = 1/K$

Scheme II

$$F + L \underset{k_d}{\overset{k_a}{\rightleftharpoons}} FL$$

$$F + S \underset{k_{-1}}{\overset{k_1}{\rightleftharpoons}} FS \xrightarrow{k_2} FP \xrightarrow{k_3} F + P$$

$$FS + L \underset{k'_{-1}}{\overset{k'_1}{\rightleftharpoons}} FLS \xrightarrow{k'_2} FLP \xrightarrow{k'_3} FL + P$$

(from paper I (Thusius et al., 1976) we have the estimate $K^{-1} = 1.1$ mM). The nonlinear fitting of the Michaelis constants to eq 7 gave $e = 1.26$ (± 0.03) mM, $f/e = 0.008$ (± 0.004) mM^{-1}, and $c = 0.040$ (± 0.009) mM^{-1}. We then calculate $f = 0.010$ (± 0.005). It will be noted that the value of c derived from V_{max}^{app} is within the 90% confidence interval of the estimate obtained from the K_m^{app} data.

Replots assuming the above parameters (Figure 2) demonstrate that Scheme I does in fact accurately account for the oxalate dependence of V_{max}^{app}/C_F^0 and K_m^{app} at all concentrations investigated.

In principle the following constants can be evaluated from the empirical parameters of the least-squares analysis: $K_m = e$; $k_2' = b/(c - b/k_d)$; $k_2 = a$; $k_1 = b/f$; $K_m' = 1/(c - b/k_d)$; $k_{-1} = eb/f - a$, where $k_d = 15$ sec^{-1} from our temperature-jump experiments (Thusius et al., 1976). Unfortunately, the quantities $(c - b/k_d)$ and $(eb/f - a)$ represent small differences between two large numbers; due to experimental uncertainties in the empirical constants, only limiting values could be estimated for K_m', k_2', and k_{-1}. Although the specific rate constants for ternary complex formation and dissociation cannot even in principle be calculated from the data at hand, an accurate lower limit for k_1' is given by the relation:

$$k_1' \geq k_2'/K_m' = b \qquad (8)$$

In summary, we find $k_1 = 1.1$ (± 0.5) $\times 10^5$ M^{-1} sec^{-1}, $k_{-1} \lesssim 250$ sec^{-1}, $K_m = 1.26$ (± 0.03) mM, $K_m' \gtrsim 60$ mM, $k_2 = 81$ (± 1) sec^{-1}, $k_1' \geq 1.1$ (± 0.2) $\times 10^3$ M^{-1} sec^{-1}, and $k_2' \gtrsim 60$ sec^{-1}.[2]

The above steady-state kinetic analysis together with the thermodynamic and temperature-jump results of the preceding paper (Thusius et al., 1976) provide a simple, self-consistent picture of oxalate inhibition. However, it will be brought out in the Discussion that Scheme I cannot account for recent rate data on the enzymatic oxidation of L-lactate deuterated at C-2 (Lederer, 1974). In an attempt to rationalize this new result, we considered an expanded mechanism (Scheme II) in which an additional species is introduced between the initial enzyme–substrate complex and product.

It can be shown that making the steady-state assumption for all intermediates in the above mechanism yields a hyperbolic rate law (eq 1), where the apparent maximum rates and Michaelis constants are again given by eq 3 and 4. Therefore, both Schemes I and II are consistent with the data of Figures 1 and 2, but now the definitions of the empirical constants are: $a = k_3/[1 + (k_3/k_2)]$, $b = \{k_3/[1 + (k_3/k_2)]\}\{k_1'/[1 + (k_{-1}'/k_2')]\}$, $c = \{k_3/[1 + (k_3/k_2)]\}\{k_1'/[1 + (k_{-1}'/k_2')]\}\{[(1/k_2') + (1/k_3') + (1/k_d)]\}$, $d = 1/K$, $e = [1 + (k_2/k_{-1})]/\{(k_1/k_{-1})[1 + (k_2/k_3)]\}$, $f = \{k_3/[1 + (k_3/k_2)]\}\{k_1'/[1 + (k_{-1}'/k_2')]\}\{1/k_1\}$. Interestingly, k_1 is given by the same expression as for Scheme I:

$$k_1 = (b/f) = 1.1 \times 10^5\ M^{-1}\ \text{sec}^{-1}$$

and $b = 1 \times 10^3$ M^{-1} sec^{-1} remains a lower limit for k_1'. We also note that:

$$k_{-1}/[1 + (k_2/k_3)] = [(eb/f) - a] \leq 250\ \text{sec}^{-1}$$

Discussion

The catalytic oxidation of L-lactate to pyruvate by cytochrome b_2 in the presence of acceptor molecules has been studied by numerous authors (Morton and Sturtevant, 1964; Suzuki and Ogura, 1970; Iwatsubo et al., 1968) with the view of defining the path of electron transfer from substrate to acceptor via the heme and flavine prosthetic groups of the enzyme. Our own steady-state kinetic measurements relate only marginally to this question, since we have worked in the limit of high acceptor concentration; it is known that above 100 μM ferricyanide all enzyme forms are present in their oxidized states (Morton and Sturtevant, 1964), and the initial rate of substrate disappearance becomes independent of acceptor concentration (Table I).

Earlier work described the oxalate inhibition of cytochrome b_2 activity as competitive (Baudras, 1965; Somlo and Slonimski, 1966). The more detailed study presented here clearly shows that oxalate exhibits mixed competitive–noncompetitive behavior with both V_{max}^{app} and K_m^{app} varying with oxalate concentration. The dependence of the initial rates on L-lactate and oxalate concentration can be rationalized in terms of a minimal scheme involving formation of a ternary complex between enzyme, substrate, and inhibitor. At the molecular level, Scheme I implies that the oxalate and lactate sites overlap, but not to the extent where binding becomes mutually exclusive. As found directly for oxalate (Thusius et al., 1976) the second-order rate constant for lactate binding deduced from steady-state measurements is unusually small, 1×10^5 M^{-1} sec^{-1}. We also note that the lower limit $k_1' \geq 1 \times 10^3$ M^{-1} sec^{-1} (i.e., k_2'/K_m') is much smaller than for "typical" enzyme substrate systems (Peller and Alberty, 1959).

Recently, Lederer (1974) measured K_m and V_{max} for the cytochrome b_2 catalyzed oxidation of C-2 deuterated L-lactate by ferricyanide. Deuteration was found to decrease V_{max} by a factor of 5, while K_m remained essentially unchanged. It seems unlikely that deuteration would greatly modify the rate of initial noncovalent complex formation, and one may conclude, as did Lederer (1974), that proton abstraction from substrate is rate limiting. In terms of Scheme I, the isotope effect is interpreted as originating entirely in k_2, leaving k_1 and k_{-1} unchanged. It then follows from eq 5 that the insensitivity of K_m to deuteration requires $k_{-1} \gg k_2$. The conclusion that k_{-1} is much larger than k_2 is, however, at variance with our own results; the steady-state kinetics of oxalate inhibition imply $k_{-1}/k_2 < 3$. Therefore, the simple Michaelis–Menten model does not satisfactorily account for both oxalate inhibition *and* the deuterium isotope effect reported by Lederer.

The next most straightforward mechanism explicitly includes the transformation of the Michaelis complex to enzyme-bound product before dissociation to free pyruvate and enzyme:

$$F + S \underset{k_{-1}}{\overset{k_1}{\rightleftharpoons}} FS \xrightarrow{k_2} FP \xrightarrow{k_3} F + P \qquad (9)$$

[2] The limiting values are based on the standard errors given in the text. For c, we chose 0.06 ± 0.007.

If species FS also binds oxalate, the mechanism for oxalate inhibition is given by Scheme II. We have shown that this model rationalizes the data of Figures 1 and 2. As found for Scheme I, the rate constant k_1 is $1.1 \times 10^5\ M^{-1}\ \text{sec}^{-1}$. In addition, the expanded scheme can account for the results with deuterated substrate. In the absence of oxalate, the initial rates are characterized by the parameters:

$$V_{max}/C_F^0 = k_3/[1 + (k_3/k_2)] \quad (10a)$$

$$K_m = (k_3/k_1)[(k_{-1} + k_2)/(k_3 + k_2)] \quad (10b)$$

If we take the process $FP \rightarrow F + P$ to represent the elementary step for product release, it is reasonable to assume that k_3 will be relatively insensitive to lactate deuteration, and that the observed decrease in V_{max} originates in k_2 alone. The maximum isotope effect for the rupture of a carbon–hydrogen bond is about 8.

One can then calculate that the fivefold decrease in V_{max} requires $k_3/k_2 \gtrsim 1$. There are two possible interpretations of the observation that V_{max}, but not K_m, exhibits an isotope effect:

$k_3, k_{-1} \gg k_2$	$k_{-1} \simeq k_2 \simeq k_3$
$k_2 = V_{max}/C_F^0 = 80\ \text{sec}^{-1}$	$k_3 \simeq 2V_{max}/C_F^0 = 160\ \text{sec}^{-1}$
case A	case B

In case A, the modification of k_2 is not expressed in K_m due to the fact that the latter is dominated by k_3 and k_{-1}. In case B, the isotope effect is cancelled by nearly equal contributions from the numerator and denominator of eq 10b.

The empirical constants determined in fitting the oxalate inhibition data seem to favor case B. From the relation shown in eq 11, we may conclude that for case A, $k_{-1} \leqslant 250\ \text{sec}^{-1}$. However, since at the same time $k_2 = 80\ \text{sec}^{-1}$, the condition $k_{-1} \gg k_2$ is not satisfied. On the other hand, the relation $k_2 \simeq k_3$ in case B yields (from eq 11) $k_{-1} \leqslant 500\ \text{sec}^{-1}$, which is consistent with the condition $k_{-1} \simeq k_2 \simeq k_3 \simeq 160\ \text{sec}^{-1}$.

$$k_{-1} = [(eb/f) - a]\ [1 + (k_2/k_3)] \leqslant 250[1 + (k_2/k_3)] \quad (11)$$

Relevant to our results is the earlier work of Hinkson and Mahler (1963a) who carried out a detailed investigation of the effect of lactate, pyruvate, and acceptor concentration on the steady-state kinetics of cytochrome b_2 catalysis. For substrate binding these authors conclude that $k_1 = 1\text{–}2 \times 10^5\ M^{-1}\ \text{sec}^{-1}$, $k_{-1} = 80\text{–}110\ \text{sec}^{-1}$, and $K_s = k_{-1}/k_1 = 0.6$ mM at pH 7.5 and 20°C. The fact that these parameters are not very sensitive to acceptor structure (Hinkson and Mahler, 1963a), the good agreement with our own values of k_1 and the upper limit of k_{-1}, as well as the precedent for slow ligand binding provided by the oxalate temperature-jump results (Thusius et al., 1976), argue that k_1 and k_{-1} do in fact refer to an elementary binding step, prior to enzyme reduction. Iwatsubo and Capeillère (1967) have noted that other flavoprotein–substrate interactions may also be characterized by second-order rate constants much smaller than diffusion-controlled values.

Conceivably the small value for k_1 arises from severe steric restrictions, as implied for oxalate binding (Thusius et al., 1976). This proposal could be tested experimentally by determining activation parameters for lactate binding in oxalate inhibition experiments, which would require the accurate determination of the temperature dependence of V_{max}^{app} and K_m^{app} over the widest possible temperature and concentration range.

It is also noteworthy that product dissociation is partially rate determining for the overall reaction of eq 9. This is not unreasonable since the interaction between oxalate and oxidized cytochrome b_2, which is presumably a simple association–dissociation, is characterized by a dissociation rate constant an order of magnitude smaller than V_{max}/C_F^0 (Thusius et al., 1976).

A recurring question in the study of enzyme catalysis is whether the equilibrium constant for initial enzyme–substrate complex formation, K_s, is equal to the experimental Michaelis constant K_m. While our results and those of Hinkson and Mahler (1963a) cannot be rationalized by assuming $K_m \equiv K_s$, they do imply that K_s cannot be much smaller than K_m ($K_s \simeq 0.5K_m = 0.6$ mM). Given the fact that other α-hydroxy acids (including D-lactate) have cytochrome b_2 inhibition constants (K_i) of $\sim$1 mM (Hinkson and Mahler, 1963b), no unusually strong interaction is suggested for L-lactate binding to the oxidized enzyme.

Iwatsubo and Capeillère (1967) attempted to detect the binding of substrate to oxidized cytochrome b_2 by measuring the effect of L-lactate on initial rates of enzyme denaturation in urea. Protection by the substrate was consistent with $K_s = 0.03$ mM, which is 40-fold smaller than K_m. From the second-order rate constant k_1 it was concluded that $k_{-1} = 2\ \text{sec}^{-1}$ (Iwatsubo and Capeillère, 1967). Clearly these earlier results are in disagreement with our conclusion that $K_s \simeq 0.6$ mM and $k_{-1} \gtrsim 100\ \text{sec}^{-1}$.

This discrepancy may well originate from complications arising in the protection experiments. From the known rate of lactate reduction in urea (Iwatsubo and Capeillère, 1967) and the concentrations of substrate and enzyme in the protection experiments, we calculate that most of the enzyme was reduced during the mixing of reagents, prior to urea denaturation. Since K_s refers to the binding of lactate to the oxidized enzyme, the denaturation experiments do not lend themselves to a straightforward interpretation concerning the ratio K_s/K_m and the value of k_{-1}.

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Soluble Tri- and Dipeptidases in *Escherichia coli* K-12†

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ABSTRACT: As part of a study of the metabolic role of peptidases in *Escherichia coli* K-12, cell extracts were examined for the presence of three enzymes originally identified [Sussman, A. J., and Gilvarg, C. (1970), *J. Biol. Chem. 245,* 6518] in extracts of the lysine auxotroph AS013 by virtue of their activity toward lysine homopolymers. It has now been shown that the activity ascribed to a Co^{2+}-dependent dilysine-specific enzyme is a function of the strain K-12 dipeptidase DP, a metal-dependent enzyme active toward a variety of dipeptides, and that the activity ascribed to a trilysine-specific enzyme is a function of the strain K-12 tripeptidase TP, an aminopeptidase capable of hydrolyzing substrates in the series X-Gly-Gly, X-Gly-X, and X-Leu-Gly (where X is Leu or Met) but devoid of activity toward dipeptides. The third enzyme, an oligopeptidase not previously observed in strain K-12, was found to include among its substrates not only di- and trilysine but other di- and tripeptides that are hydrolyzed by the di-and tripeptidase as well as by aminopeptidases L and AP; the aminopeptidases, however, lack activity toward di- and trilysine. The absence of oligopeptidase activity from extracts of strain AJ005, a "peptidase-deficient mutant" derived from strain AS013 by Sussman and Gilvarg, has been confirmed, and strain AJ005 has been shown to contain all the other peptidases known to be present in strain K-12. Possible functions of the oligopeptidase are proposed on the basis of its observed activity in vitro and of the differences between the growth responses of strains AJ005 and AS013 in various media. Some general aspects of peptide metabolism are discussed with emphasis on the use of peptidase-deficient mutants in the study of this problem, and methods that may prove helpful in the isolation of such mutants are suggested.

Evidence that bacterial enzymes catalyzing the hydrolysis of small peptides participate in a variety of metabolic processes has been accumulating since the role of peptidases in the utilization of exogenous peptides as growth factors and in the destruction of toxic peptides was recognized in 1950 (Fruton and Simmonds, 1950). Subsequent work in several laboratories (summarized by Sussman and Gilvarg, 1971; Simmonds, 1972) has provided ample support for these two functions of intracellular peptidases in *Escherichia coli* K-12. During the 1960's, further interest in bacterial peptidases was stimulated by the realization that bacterial protein biosynthesis may require the participation of a methionine aminopeptidase, and *E. coli* K-12 has been shown to contain several enzymes capable of effecting the removal of an amino-terminal methionine from tri- or dipeptides (Simmonds, 1972)[1] in addition to the "aminopeptidase I" crystallized by Vogt (1970). Whether any of these strain K-12 peptidases, or the "ribosomal-bound aminopeptidase" (Matheson et al., 1970) and the dipeptidases (Brown, 1973; Hayman et al., 1974) purified from *E. coli* strain B, are specifically concerned in protein biosynthesis remains uncertain (cf., Vogt, 1970; Matheson and Dick, 1970; Brown, 1973; Johnson and Brown, 1974). The suggestion has been made that a primary function of such enzymes is their participation in protein turnover, i.e., as catalysts for the hydrolysis of small peptides arising from the degradation of cellular proteins which provides amino acids for the formation of new protein molecules (Simmonds, 1970). Rapid degradation of protein in nongrowing bacteria was first reported by Mandelstam and Halvorson in 1960, and later studies by several investigators (summarized by Payne, 1972b; Goldberg et al., 1974) support the view that intracellular proteolysis may not only supply amino acids for the synthesis of new enzymes in starved *E. coli* cells but also provides a mechanism for the elimination of abnormal cell constituents formed as the result of mutations or mistakes in transcription or translation (Goldberg et al., 1974; Goldberg and Dice, 1974).

Although the enzymic activity of crude *E. coli* extracts toward di- and tripeptides appears to be sufficient to account for the relatively high rates of protein degradation to amino acids which have been observed in starved cells (Payne, 1972b), the existence of several peptidases showing overlapping substance specificity raises the question of whether the full complement of peptidases present in a wild-type strain is essential for this process as well as for cell growth and multiplication. Resolution of this problem would most easily be accomplished by studies on amino acid auxotrophs lacking one or more of the "wild-type peptidases". Most attempts to produce such mutants have been unsuccessful (Payne, 1972b; F. Taylor and S. Simmonds, unpublished data), and only one peptidase auxotroph of strain K-12 has been described: a mutant derived from the lysine

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[1] S. Simmonds, C. G. Fletterick, and T. Kubaska, manuscript in preparation.