Transient-State Kinetic Analysis of Urocanase†

Larry H. Matherly, Kenneth A. Johnson, and Allen T. Phillips*

ABSTRACT: Transient-state kinetics were studied to determine the participation of bound NAD^+ (nicotinamide adenine dinucleotide) in catalysis by urocanase from *Pseudomonas putida*. An enzyme-bound species fluorescing at 420 nm when excited at 334 nm was formed upon mixing urocanase either with its substrate, urocanic acid, or with a competitive inhibitor, imidazolepropionic acid. The hyperbolic concentration dependence of the rate of formation of these fluorescent intermediates suggested a pathway of the type

$$E + S \overset{1}{\rightleftharpoons} ES \overset{2}{\rightleftharpoons} ES'$$

For urocanic acid, the first step involved a nonfluorescent rapid binding equilibrium between enzyme and substrate, with an association constant (K_1) of 2.5×10^3 M^{-1}. This step was followed by a slower fluorescence transition with a first-order rate constant (k_2) of 180 s^{-1}. The turnover number for product formation from urocanate was 7.6 s^{-1}, and therefore, the fluorescent species forms at a rate that would allow it to be an intermediate in the reaction path leading to product. The kinetic constants for imidazolepropionate, which formed a fluorescent species by a parallel pathway, were K_1 of 1.6×10^2 M^{-1} and k_2 of 46 s^{-1}. The temperature dependence of the apparent rate constants (k_2) for formation of the urocanate and imidazolepropionate fluorophores was also determined. The Arrhenius activation energies were 16.3 kcal mol^{-1} (68 kJ mol^{-1}) and 19.3 kcal mol^{-1} (81 kJ mol^{-1}) for urocanate and imidazolepropionate, respectively. These data indicate that very similar reactions give rise to the two fluorescent species and thus support the formation during catalysis of an NAD–urocanate adduct analogous to the covalent complex involving imidazolepropionate and NAD bound to urocanase [Matherly, L. H., DeBrosse, C. W., & Phillips, A. T. (1982) *Biochemistry* (preceding paper in this issue)].

The mechanism for the urocanase-catalyzed conversion of urocanic acid to 5-hydroxyimidazolepropionic acid, the enol form of oxoimidazolepropionic acid, has been the focus of a number of studies aimed at understanding the role of NAD^+ (nicotinamide adenine dinucleotide) in the reaction process. While urocanase (EC 4.2.1.49) has an absolute catalytic requirement for a tightly bound NAD^+ (Egan & Phillips, 1977; Keul et al., 1979), NADH (reduced NAD) is apparently not formed during catalysis, as shown by the absence of a direct transfer of a hydride ion in deuterium labeling experiments (Egan et al., 1981) and by the demonstration that the enol tautomer of oxoimidazolepropionic acid is the reaction product (Kaeppeli & Retey, 1971; Matherly & Phillips, 1981).

The substrate analogue imidazolepropionate is not hydrated by urocanase, yet it binds to the enzyme to generate a chromophoric species that has an absorption spectrum resembling that of bound NADH. However, this material can be readily distinguished from the reduced pyridine nucleotide by its fluorescence emission spectrum and by enzymatic assay and has been characterized as a covalent addition product of imidazolepropionate with the nicotinamide ring of NAD on urocanase (Matherly et al., 1982). A similar fluorescent intermediate involving urocanate has not been demonstrated, yet the observation that both imidazolepropionate and urocanate serve as substrates for the urocanase-catalyzed rapid exchange of the imidazole 5 hydrogen with solvent (Egan et al., 1981) argues for a similarity in the binding of these compounds.

In the present study we addressed the question of the catalytic role of the urocanase NAD^+ by transient-state kinetic methods. Our results show that the substrate, urocanate, forms a fluorescent enzyme-bound intermediate by a pathway that parallels the formation of the enzyme-bound NAD–imidazolepropionate adduct. The kinetic similarities of the two reactions suggest that the fluorescent intermediate observed during catalysis is an NAD–urocanate adduct analogous to the better characterized NAD–imidazolepropionate adduct. Moreover, the rate of formation of this intermediate is sufficiently rapid to allow it to be an obligatory intermediate in the catalytic pathway.

Experimental Procedures

Urocanase was purified to homogeneity from *Pseudomonas putida*, ATCC 12633, by the procedure of George & Phillips (1970). Urocanic acid was purchased from Sigma Chemical Co. Imidazolepropionic acid was prepared by catalytic hydrogenation of urocanic acid (Phillips et al., 1977). Other chemicals were obtained from commercial sources. Conventional spectrophotometric determinations of urocanase activity and protein content were performed as described by George & Phillips (1970).

The kinetics of the pre steady state were obtained with a stopped-flow apparatus constructed in this laboratory (K. A. Johnson, unpublished results). The path length of the observation cell was 2 mm and required a sample volume of 100 μL from each syringe. The ball-type mixer (Berger, 1978) was obtained from Research Instruments and Manufacturing Co., San Diego, CA. The dead time of the apparatus was 1 ms. Fluorescence was excited at 334 nm (10-nm band-pass) and observed at 420 nm (20-nm band-pass) at 90° to the incident beam by using interference filters obtained from Omega Optical, Inc., Brattleboro, VT. The samples and the observation cell were thermostated by using a circulating water bath, and the temperature in the sample chamber was measured with a thermocouple. The temperature was maintained at 20 °C in all experiments except for the temperature study. The data were collected with an On-Line Instrument Systems 3820 data acquisition system and were fit to a single expo-

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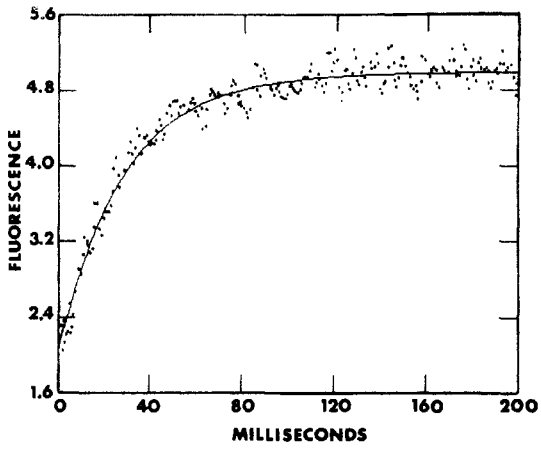

FIGURE 1: Kinetics of the fluorescence transient for NAD–imidazolepropionate formation. Fluorescence was recorded as a function of time following the mixing of urocanase with imidazolepropionate (16 mM final concentration). Each dot represents a single data point with 250 data points/trace. The line is the best fit to a single exponential. Fluorescence is expressed in arbitrary units.

nential by the method of moments (Dyson & Isenberg, 1971). For the experiments with imidazolepropionate, the final protein concentration after mixing was 0.5 mg mL^{-1} and for urocanate 1.5 mg mL^{-1}; the buffer used in all experiments was 50 mM tris(hydroxymethyl)aminomethane hydrochloride (Tris-HCl).

Results and Discussion

Kinetics of Formation of NAD–Imidazolepropionate. Incubation of urocanase with the substrate analogue imidazolepropionate results in the formation of an enzyme-bound chromophore that has been characterized as a covalent addition complex between the analogue and the nicotinamide portion of the active site NAD (Matherly et al., 1982). This species has a fluorescence emission spectrum with a maximum at 420 nm when excited at 330 nm. Figure 1 illustrates the time course of the increase in fluorescence corresponding to the formation of the NAD–imidazolepropionate complex. The data in Figure 1 were fit to a single exponential of the form

$$\Delta\phi = 1 - e^{-\lambda t} \quad (1)$$

where $\Delta\phi$ equals the change in fluorescence and t represents the data recording time. The best fit for the apparent pseudo-first-order rate constant (λ) at 16 mM imidazolepropionate was 35 s^{-1} (Figure 1).

The measurements of the rate of the fluorescence change as a function of imidazolepropionate concentration are summarized in Figure 2. These data show a hyperbolic concentration dependence, suggesting at least a two-step pathway leading to the formation of the fluorescent NAD–imidazolepropionate complex:

$$E + I \overset{1}{\rightleftharpoons} EI \overset{2}{\rightleftharpoons} EI' \quad (2)$$

where I represents the inhibitor, imidazolepropionate, and EI′ designates the fluorescent NAD–imidazolepropionate adduct.

If a rapid equilibrium is assumed for step 1, the rate of formation of the fluorescent species is given by

$$\lambda = K_1 k_2 [I]/(1 + K_1[I]) + k_{-2} \quad (3)$$

The line drawn in Figure 2 is the best fit of the data according to eq 3 with $K_1 = 1.6 \times 10^2$ M^{-1}, $k_{-2} \leq 1$ s^{-1}, and $k_2 = 46$ s^{-1}. Thus, the rate-limiting step for the formation of the enzyme-bound NAD–imidazolepropionate adduct was relatively fast (46 s^{-1}) at saturating imidazolepropionate concentration.

We have assigned the entire observed fluorescence change to the formation of the NAD–imidazolepropionate complex

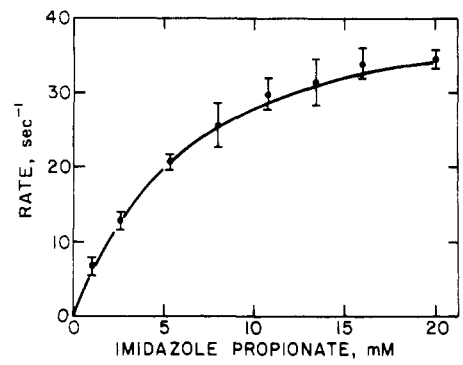

FIGURE 2: Imidazolepropionate concentration dependence for transient rates of NAD–imidazolepropionate formation. Fluorescence rates were measured as described in Figure 1 with final imidazolepropionate concentrations between 0.25 and 20 mM. The data are presented as the mean value ± standard deviation of three to four traces. The line represents the best fit to a hyperbola.

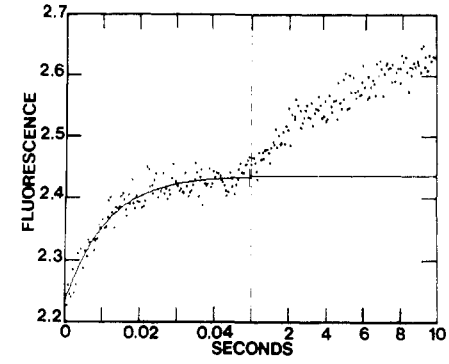

FIGURE 3: Kinetics of pre-steady-state and steady-state fluorescence with urocanate. Fluorescence was recorded as a function of time following the mixing of urocanase with urocanate (1 mM final concentration). The first 125 data points were collected over the first 50 ms while the second 125 data points were recorded up to 10 s. The line is the best fit of the transient rate to a single exponential.

(EI′) occurring in step 2 of eq 2. The question of whether there was a change in fluorescence attributable to binding (step 1) was addressed in two ways. First, it was shown by mixing enzyme with buffer in the stopped-flow apparatus that the observed reaction with imidazolepropionate began at a fluorescence level equal to that of the free enzyme. Therefore, no fast signal attributable to step 1 was lost in the dead time of the apparatus. Second, the fluorescence transient was evaluated as to whether it deviated from a single exponential. In all cases, the fit to a single exponential was good, and no systematic deviation was detected. These results indicate that the fluorescence change occurred as a single rate process and was attributable to the reaction that produced the NAD–imidazolepropionate adduct (step 2, eq 2).

Kinetics of the Formation of a Fluorescent Intermediate with Urocanate. A pre-steady-state increase in fluorescence was also observed following the mixing of urocanic acid with urocanase (Figure 3). In contrast to the case with imidazolepropionate, however, there was a further increase in fluorescence observable on a longer time scale (Figure 3). This linear increase in fluorescence with time represents the accumulation of product, the enol tautomer of oxoimidazolepropionic acid, which exhibits excitation and emission maxima at 330 and 430 nm, respectively (Matherly & Phillips, 1981). The maximum rate of this steady-state turnover, 7.6 s^{-1} (50 mM Tris, pH 7.5; 20 °C), was sufficiently slow compared to the rate of pre-steady-state transient so that the two processes were well separated in time. A good fit to a single exponential was obtained for the pre-steady-state transient, giving a rate

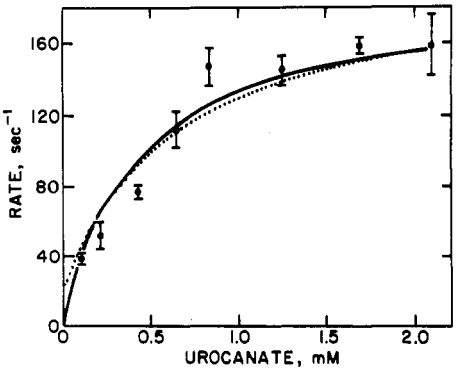

FIGURE 4: Urocanic acid concentration dependence for pre-steady-state fluorescence transients. Fluorescence was recorded over a 100-ms period (250 data points) with final concentrations of urocanic acid between 0.1 and 2.4 mM. The data are presented as the mean value ± standard deviation of three to four traces. The solid line represents a calculated fit to eq 3 in which $K_1 = 2.6 \times 10^3$ M^{-1}, $k_2 = 185$ s^{-1}, and $k_{-2} = 0$; the dotted line was calculated on the basis of $K_1 = 1.5 \times 10^3$ M^{-1}, $k_2 = 180$ s^{-1}, and $k_{-2} = 20$ s^{-1}.

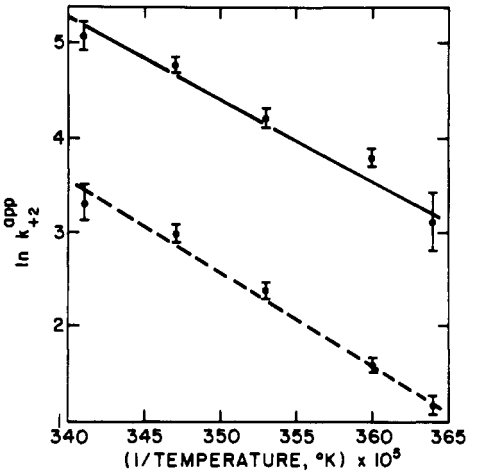

FIGURE 5: Temperature dependence for fluorescence transients. Fluorescence was determined as described in Figures 1 and 3 by using 21.0 mM imidazolepropionate (---) or 2.1 mM urocanic acid (—) between 2 and 20 °C. The data are reported as the mean value ± standard deviation of three to four traces.

of 90 s^{-1} at 1 mM urocanate (Figure 3).

The concentration dependence of the rate of the fluorescence transient is shown in Figure 4. As in the case described for imidazolepropionate, the data are indicative of a minimum two-step reaction pathway leading to the formation of the fluorescent species. The data could be fit equally well to eq 3 by assuming $k_{-2} = 0$ to give $K_1 = 2.6 \times 10^3$ M^{-1} and $k_2 = 185$ s^{-1} (Figure 4, solid line) or by the set of constants $K_1 = 1.5 \times 10^3$ M^{-1}, $k_{-2} = 20$ s^{-1}, and $k_2 = 180$ s^{-1} (Figure 4, dotted line). In either case the data demonstrate that the reaction leading to the formation of the fluorescent intermediate at saturating substrate concentration is very fast compared to the steady-state turnover rate (7.6 s^{-1}).

Amplitude measurements revealed that the only loss in signal at saturating substrate concentration could be attributed to the known dead time of the apparatus and the rate of the observed reaction. Since the observed reaction could be adequately fit to a single exponential, there was no apparent change in fluorescence accompanying the substrate binding step, and the observed fluorescence change occurred kinetically as a single reaction step.

The data obtained with urocanate, taken alone, might be interpreted to suggest that the pre-steady-state transient represents the accumulation of the product on the enzyme, with product release being the rate-limiting step in the steady state. However, the similar fluorescence transient observed with imidazolepropionate, which can form no product, argues that the fluorescent species formed in the presence of urocanate represents a new intermediate in the pathway distinct from the fluorescent product. Accordingly, the minimum pathway to account for the results can be described as

$$E + S \overset{1}{\rightleftharpoons} ES \overset{2}{\rightleftharpoons} ES' \overset{3}{\rightleftharpoons} EP \overset{4}{\rightleftharpoons} E + P \qquad (4)$$

where S represents the substrate, urocanate, ES′ designates the fluorescent intermediate analogous to EI′ (eq 2), and P refers to the product.

Even though both the intermediate and the product, the enol form of oxoimidazolepropionic acid, are fluorescent, we have not detected a change in fluorescence attributable to the formation of product from ES′ (step 3 in eq 4). That is, the fluorescence transient fits a single exponential, and there was a linear increase in fluorescence from the steady-state release of product, which occurred with no apparent lag following the transient. This observation can be explained in two ways: (1) ES′ and EP exhibit approximately equal fluorescence quantum yields that cannot be distinguished under the conditions employed, and k_4 is the rate-limiting step in the steady state with $k_2 > k_3 > k_4$; (2) k_3 is the rate-limiting step in the steady state and $k_2 > k_3 < k_4$. We cannot distinguish these alternatives and we have no kinetic evidence for additional intermediates following ES′. However, in either case, the observed accumulation of the fluorescent intermediate implies that step 3 is slower than step 2, given the reaction pathway described by eq 4. Since it is likely that subsequent intermediates do exist (Egan et al., 1981) and that product release may be rate limiting, we have tentatively assigned the value of k_4 as 7.6 s^{-1}, the steady-state turnover number.

Estimation of Arrhenius Activation Energies for Fluorescent Transients. The kinetic data obtained with urocanate and imidazolepropionate indicate that both undergo similar reactions with the enzyme to form fluorescent intermediates. In order to investigate further the similarity of these reactions, we determined the temperature dependence of the maximum rate of the fluorescence transient (k_2) for each species. For both urocanate and imidazolepropionate, the fluorescent transients were adequately fit by single exponentials at all temperatures. An Arrhenius plot summarizing the relationship between temperature and the apparent first-order rate constants at saturating substrate concentration is presented in Figure 5. The energies of activation, calculated from the slopes of the lines in Figure 5, are 16.3 kcal mol^{-1} (68 kJ mol^{-1}) and 19.3 kcal mol^{-1} (81 kJ mol^{-1}) for urocanate and imidazolepropionate, respectively. The closeness of these numbers provides additional support that the fluorescent species formed by each compound result from similar reactions.

In the preceding paper (Matherly et al., 1982), we described the isolation and characterization of the oxidized form of an addition complex between the urocanase NAD and imidazolepropionate. In the present study, we have shown that urocanate and imidazolepropionate each undergo parallel reactions with urocanase to form a fluorescent species and that these reactions have comparable rates and activation energies. These experiments provide the first direct evidence that urocanase catalysis proceeds with the formation of a transient intermediate involving the active site NAD. The kinetic data with imidazolepropionate and urocanate taken together offer considerable support for the postulate that a covalent pyridine nucleotide–substrate complex is an obligatory catalytic intermediate (Egan et al., 1981).

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Positive Regulation of Activation of Plasminogen by Urokinase: Differences in K_m for (Glutamic acid)-plasminogen and Lysine-plasminogen and Effect of Certain α,ω-Amino Acids†

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ABSTRACT: The kinetics of activation of human Glu- and Lys-plasminogens by human urokinase and the effect of lysine on the kinetics of activation are analyzed in a simple assay with the active site titrant for plasmin 3′-(4-guanidinobenzoyloxy)-6′-hydroxyspiro[isobenzofuran-1(3*H*),9′-[9*H*]-xanthen]-3-one. The K_m for the activation of Glu-plasminogen is 200 μM and for Lys-plasminogen is 12 μM, while the k_{cat} values are 1.48 s^{-1} and 1.89 s^{-1}, respectively. In the presence of 0.1 M lysine, the K_m for the activation of Glu-plasminogen decreases 10-fold to 24 μM and for Lys-plasminogen is 7.5 μM, while the k_{cat} values are 1.78 s^{-1} and 1.62 s^{-1}, respectively. Lysine is a competitive inhibitor of urokinase with a K_i of 0.1 M. These data explain some of the well-documented effects of certain α,ω-amino acids on the activation of plasminogen and are relevant to their use in clinical therapy. Glu-plasminogen is not as activatable as Lys-plasminogen, because its K_m is 10-fold higher. Lysine renders Glu-plasminogen more activatable, because it lowers the K_m 10-fold to that of Lys-plasminogen. High concentrations of lysine inhibit the activation of both Glu- and Lys-plasminogens, because it is a competitive inhibitor of urokinase. Thus, at low concentrations certain α,ω-amino acids are fibrinolytic whereas at high concentrations they are antifibrinolytic. These experiments have led us to propose a new model for the positive regulation of fibrinolysis in vivo. In plasma the concentration of Glu-plasminogen is 100-fold lower than its K_m for activation by urokinase. Thus, even in the presence of urokinase little or no activation to plasmin occurs. Upon formation of a fibrin clot, Glu-plasminogen binds at its lysine binding sites to the clot [Wiman, B., & Wallén, P. (1977) *Thromb. Res. 10*, 213-222]. This lowers the K_m for activation 10-fold so that plasmin now begins to form. Once the clot is dissolved by plasmin, the lysine binding sites in Glu-plasminogen are no longer bound to fibrin, and thus the K_m for activation is increased 10-fold. Requiring for activation of plasminogen the simultaneous presence of both a plasminogen activator and a plasmin substrate to which plasminogen is bound ensures that the formation of plasmin occurs quickly and only where and when it is needed.

The fibrinolytic system is highly regulated (Kline & Reddy, 1980). The basic components are plasminogen, plasminogen activators, and plasmin. Under normal physiological conditions the basic components of the fibrinolytic system are present in the blood. How, on the one hand, can plasminogen and plasminogen activators coexist without all the plasminogen being converted to plasmin and yet, on the other hand, how can plasminogen be converted to plasmin at a fibrin clot? Here we address these questions by analyzing the kinetics of activation of two forms of plasminogen by urokinase in the absence and presence of L-lysine. By implication, the data indicate how fibrin may regulate plasminogen activation.

Native plasminogen consists of a single polypeptide chain composed of 790 amino acids, including an NH_2-terminal glutamic acid (Wallén & Wiman, 1972). Plasmin is formed upon proteolytic cleavage of a single arginyl–valyl bond 560 amino acids from the NH_2-terminal end of Glu-plasminogen[1] (Robbins et al., 1967). The resulting two polypeptides, the 560 amino acid A chain and the 230 amino acid B chain, are held together by a disulfide bridge. The serine and histidine of the catalytic site are located in the B chain (Groskopf et al., 1969). Plasmin can catalytically cleave native Glu-plasminogen at the lysyl–lysyl bond at position 76–77 from the NH_2 terminal, yielding a small preactivation peptide and a 714 amino acid form of plasminogen called Lys-plasminogen

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[1] Abbreviations: FMGB, 3′-(4-guanidinobenzoyloxy)-6′-hydroxyspiro[isobenzofuran-1(3*H*),9′-[9*H*]xanthen]-3-one; P_i/NaCl, phosphate-buffered saline; Glu-plasminogen, native plasminogen with an NH_2-terminal glutamic acid; Lys-plasminogen, plasminogen with an NH_2-terminal lysine that is produced by plasmin cleavage of the lysyl–lysyl bond at position 76–77 of Glu-plasminogen; A chain, the largest of the two polypeptide chains of plasmin; B chain, the smaller of the two polypeptide chains of plasmin; iPr_2P-F, diisopropyl fluorophosphate; $NaDodSO_4$, sodium dodecyl sulfate; EDTA, ethylenediaminetetraacetic acid.

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