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Elementary Steps in the Reaction Mechanism of the α-Ketoglutarate Dehydrogenase Multienzyme Complex from *Escherichia coli*: Kinetics of Succinylation and Desuccinylation†

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ABSTRACT: The kinetics of the succinylation and the desuccinylation of the α-ketoglutarate dehydrogenase complex from *Escherichia coli* have been studied at 4 °C in 2 mM thiamin pyrophosphate, 2 mM $MgCl_2$, and 20 mM potassium phosphate (pH 7.0) by steady-state and quenched-flow techniques. The initial steady-state velocity for the reaction of the complex is inhibited by high concentrations of α-ketoglutarate. The data are consistent either with cooperative interactions between two catalytic sites or with the existence of an α-ketoglutarate regulatory site. The time course of the succinylation by α-ketoglutarate of the unmodified complex or the complex in which a fraction of the α-ketoglutarate decarboxylase subunits (E_1) has been inhibited with *N*-ethylmaleimide reveals a complex kinetic process. A mechanism consistent with the kinetic data is proposed in which some E_1 subunits succinylate one lipoic acid per E_1 and other E_1 subunits succinylate two lipoic acids per E_1. Furthermore, each succinylation reaction occurs via a two-step process with rate constants of 49 and 89 s^{-1} at saturating concentrations of α-ketoglutarate for the first and second steps, respectively. At long times, 13-16 mol of succinate binds per mol of unmodified complex. The stoichiometry of binding obtained with *N*-ethylmaleimide-treated complex is initially lower but approaches the same values as for the unmodified complex over the course of minutes. Coenzyme A removes the succinyl groups on the unmodified enzyme with a rate constant ≥200 s^{-1}. The results obtained suggest a limited accessibility between sites on the complex.

The α-ketoglutarate dehydrogenase complex from *Escherichia coli* contains three enzymes, α-ketoglutarate decarboxylase (E_1),[1] dihydrolipoyl transsuccinylase (E_2), and dihydrolipoyl dehydrogenase (E_3), and catalyzes the following sequence of reactions:

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[1] Abbreviations: E_1, α-ketoglutarate decarboxylase; E_2, dihydrolipoyl transsuccinylase; E_3, dihydrolipoyl dehydrogenase; CoA, coenzyme A; MalNEt, *N*-ethylmaleimide.

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$$\alpha\text{-ketoglutarate} + E_1[TPP] \rightleftharpoons CO_2 + E_1[\text{hydroxybutyryl-TPP}] \quad (1)$$

$$E_1[\text{hydroxybutyryl-TPP}] + E_2[\text{Lip-}S_2] \rightleftharpoons E_1[TPP] + E_2[\text{HS-Lip-}S\text{-succinyl}] \quad (2)$$

$$E_2[\text{HS-Lip-}S\text{-succinyl}] + CoA \rightleftharpoons E_2[\text{Lip-}(SH)_2] + \text{succinyl-CoA} \quad (3)$$

$$E_2[\text{Lip-}(SH)_2] + E_3[\text{FAD,ox}] \rightleftharpoons E_2[\text{Lip-}S_2] + E_3[\text{FAD,red}] \quad (4)$$

$$E_3[\text{FAD,red}] + NAD^+ \rightleftharpoons E_3[\text{FAD,ox}] + NADH + H^+ \quad (5)$$

where TPP, Lip-S_2, and Lip-$(SH)_2$ are thiamin pyrophosphate and oxidized and reduced lipoic acids, respectively. The stoichiometry of enzyme molecules within the complex appears to be 12:24:12 (E_1:E_2:E_3), and the complex has a molecular weight of about 2.5×10^6 (Pettit et al., 1973; Reed, 1974). The 24 E_2 subunits are arranged in octahedral symmetry and form the core of the complex; dimers of E_1 and E_3 bind to this core (DeRosier et al., 1971; Wagenknect et al., 1983). A mechanism has been postulated in which lipoic acids transfer intermediates by rotating between the three catalytic sites (Koike et al., 1963). Fluorescence resonance energy transfer measurements have shown that the catalytic sites are sufficiently close to be spanned by a single lipoic acid (Angelides & Hammes, 1979), and dynamic anisotropy experiments have demonstrated that the lipoic acids undergo motion consistent with rotation between sites (Waskiewicz & Hammes, 1982).

In this work, the kinetics of the succinylation and desuccinylation reactions of the complex (eq 1–3) have been studied by steady-state and fast-reaction techniques. The reaction of α-ketoglutarate with unmodified complex or complex in which a number of E_1 subunits have been inactivated reveals that a limited number of lipoic acids can be succinylated. A mechanism is proposed in which each succinylation occurs via a two-step process and some E_1 subunits succinylate two lipoic acids. The implications of these results for the mechanism of the overall reaction are discussed.

Experimental Procedures

Materials. *N*-Ethyl[2,3-^{14}C]maleimide (20.0 mCi/mmol) was from Amersham and was used without further purification. α-[5-^{14}C]Ketoglutarate (15.9–20.2 mCi/mmol) also was obtained from Amersham and was found to be ≥95% pure by chromatography on Bio-Rad AG 1-X4 (Von Korff, 1969). Concentrations of α-ketoglutarate solutions were determined by using the L-glutamate dehydrogenase assay (Von Korff, 1969). All other reagents were high-quality commercial grades. Unless specified, all experiments were performed at 4 °C.

Enzyme. The α-ketoglutarate dehydrogenase complex was prepared from *E. coli* (strain B) by the procedure described previously (Waskiewicz & Hammes, 1982). Protein concentrations were determined by the method of Lowry et al. (1951) using bovine serum albumin as standard with a correction factor of 0.97 and with a molecular weight for the complex of 2.5×10^6 (Pettit et al., 1973). The activity of the complex was determined spectrophotometrically at 30 °C by measuring the rate of production of NADH at 340 nm in an assay mixture consisting of 4.2 mM thiamin pyrophosphate, 3.9 mM $MgCl_2$, 5.3 mM α-ketoglutarate, 2.8 mM NAD^+, 0.2 mM CoA, and 1.1 mM dithiothreitol in 50 mM potassium phosphate (pH 8.0). The specific activity of the purified complex was 17–20 μmol of NADH min^{-1} mg^{-1}. The activity of the E_1 component of the complex was measured at 30 °C by following the rate of reduction of potassium ferricyanide at 420 nm in an assay mixture consisting of 2 mM potassium ferricyanide, 2 mM thiamin pyrophosphate, 2 mM $MgCl_2$, and 0.4 mM α-ketoglutarate in 20 mM potassium phosphate (pH 7.0).

Steady-State Kinetics. Steady-state kinetics were studied by measuring the time course of the reactions spectrophotometrically at 4 °C. The overall reaction was monitored by determining the rate of production of NADH at 340 nm by 3–5 nM enzyme complex in 0–6 mM α-ketoglutarate, 2.0 mM thiamin pyrophosphate, 2.0 mM $MgCl_2$, 3.0 mM NAD^+, 0.5 mM CoA, 1.25 mM dithiothreitol, and 20 mM potassium phosphate (pH 7.0). The reaction of the E_1 component was followed by measuring the rate of reduction of potassium ferricyanide at 420 nm by 52 nM enzyme complex in 0–6 mM α-ketoglutarate, 2.0 mM potassium ferricyanide, 2.0 mM thiamin pyrophosphate, 2.0 mM $MgCl_2$, and 20 mM potassium phosphate (pH 7.0).

Reaction of MalNEt with the Multienzyme Complex. The α-ketoglutarate dehydrogenase complex at 21.1 mg/mL was incubated with 2.0 mM nonradioactive or ^{14}C-labeled MalNEt and 1.0 mM ethylenediaminetetraacetic acid in 20 mM potassium phosphate (pH 7.0). This treatment resulted in a concomitant loss of overall and E_1 activities with a pseudo-first-order rate constant of approximately 0.3 h^{-1} at 0 °C. The reaction was stopped at various times by applying 0.3–0.4 mL of the reaction mixture to two consecutive 3.5-mL Sephadex G-25 centrifuge columns (Penefsky, 1977). The subunit location of the covalently bound MalNEt was determined by sodium dodecyl sulfate–polyacrylamide gel electrophoresis. Samples of [^{14}C]MalNEt-labeled complex (≥90% inhibited) were applied to the gels, electrophoresed, and stained as described previously (Waskiewicz & Hammes, 1982). Individual bands were cut from the gels, placed in scintillation vials, and treated with 1 mL of 90% NCS tissue solubilizer (Amersham) for 2.5 h at 50 °C. When cool, 10 mL of Aqueous Counting Scintillant (Amersham) was added to the vial, and the radioactivity was determined by liquid scintillation counting. The background radioactivity correction consisted of unlabeled portions of the gel treated in an identical manner.

Succinylation Kinetics. The time course of succinylation of the α-ketoglutarate dehydrogenase complex by α-[5-^{14}C]-ketoglutarate was studied with a quenched-flow apparatus (Akiyama, 1980) or by manual mixing. The complex and α-ketoglutarate were dissolved separately in a buffer consisting of 2 mM thiamin pyrophosphate and 2 mM $MgCl_2$ in 20 mM potassium phosphate (pH 7.0). Equal volumes of the enzyme and α-ketoglutarate solutions were then mixed and reacted for a specified length of time at 4 °C before being quenched with concentrated perchloric acid at a final acid concentration of 13%. After incubation on ice for 30–90 min, the precipitated enzyme was collected on a Whatman GF/F filter and washed sequentially with five 4-mL aliquots of ice-cold 13% perchloric acid and two 4-mL aliquots of ice-cold 95% ethanol. The filter was air-dried and placed in a scintillation vial overnight with 10 mL of scintillation fluid, shaken thoroughly, and the radioactivity was determined. A background radioactivity correction consisted of samples of α-[5-^{14}C]ketoglutarate without enzyme that were quenched and washed as described above. Typically, the complex concentration was 80 nM and the α-ketoglutarate concentration was 4.7–74 μM before quenching.

Table I: Kinetic Properties of α-Ketoglutarate Dehydrogenase Derivatives[a]

sample	overall activity (%)	E_1 activity (%)	relative k_{cat}[b]	k_{cat} (s^{-1})	α	K_m (μM)	K_2 (mM)
Cooperative Catalytic Sites Model (Equation 7)							
complex	100	100	100	145 (9)	0.497 (0.039)	18.6 (2.7)	390 (170)
MalNEt-treated complex	62	62	59	85.5 (2.9)	0.516 (0.143)	17.5 (1.7)	2.0 (0.9)
MalNEt-treated complex	33	32	36	52.6 (1.4)	0.373 (0.123)	15.4 (1.2)	3.0 (1.5)
Regulatory Site Model (Equation 8)							
complex	100	100	100	153 (14)	0.471 (0.040)	19.7 (3.4)	370 (190)
MalNEt-treated complex	62	62	56	86.0 (3.0)	0.513 (0.141)	17.6 (1.8)	2.0 (0.9)
MalNEt-treated complex	33	32	35	52.9 (1.5)	0.371 (0.122)	15.5 (1.3)	2.9 (1.5)

[a] Standard errors are given in parentheses. [b] Relative to the unmodified complex.

Desuccinylation Kinetics. Desuccinylation of the α-ketoglutarate dehydrogenase complex by CoA was studied by using the quenched-flow apparatus either by following the production of [4-^{14}C]succinyl-CoA or by monitoring the loss of succinate covalently bound to the multienzyme complex. The complex was initially succinylated by reacting it with α-[5-^{14}C]ketoglutarate for 5–10 min in 2 mM thiamin pyrophosphate, 2 mM $MgCl_2$, and 20 mM potassium phosphate (pH 7.0). CoA was dissolved in 1 mM dithiothreitol, 2 mM thiamin pyrophosphate, 2 mM $MgCl_2$, and 20 mM potassium phosphate (pH 7.0). The two solutions were mixed and reacted for a specified length of time at 4 °C before being quenched in 13% perchloric acid. The amount of succinate remaining on the complex was determined by filtering and washing the precipitated complex as described above. To determine the amount of [4-^{14}C]succinyl-CoA released, the quenched reaction mixture was centrifuged and the precipitated complex discarded. The pH of the supernatant was raised to 5–7 with KOH and the mixture centrifuged. The pellet of $KClO_4$ was discarded, and a concentrated solution of triethylammonium phosphate (pH 6.7) was added to the supernatant to a final concentration of 1 mM. At ambient temperature, the solution was loaded onto a 4.6 mm × 15 cm Altex Ultrasphere–ODS high-performance liquid chromatography column equilibrated with 1 mM triethylammonium phosphate (pH 6.7). After the α-[5-^{14}C]ketoglutarate was eluted from the column with the same buffer, [4-^{14}C]succinyl-CoA was eluted with 70% methanol. Fractions containing [4-^{14}C]succinyl-CoA were combined, and the radioactivity was determined. The background radioactivity was measured with samples of α-[5-^{14}C]ketoglutarate quenched and treated as described above. Control experiments indicated that the presence of dithiothreitol in the CoA solution did not alter the succinylation stoichiometry.

Data Analysis. The data were fit to functional forms with a nonlinear least-squares routine.

Results

Steady-State Kinetics. The dependence of the initial steady-state velocity on the concentration of α-ketoglutarate for the overall reaction of the α-ketoglutarate dehydrogenase complex at 4 °C in 20 mM potassium phosphate (pH 7.0) is shown in Figure 1. The complex does not exhibit simple Michaelis–Menten kinetics; at high substrate concentrations, the activity decreases to a lower level as the substrate concentration increases. While a slight drop in the steady-state velocity is observed when the ionic strength of the assay solution is increased with potassium phosphate, this effect is negligible over the range of α-ketoglutarate concentrations studied. Two models can account for the observed substrate inhibition. In the first model, cooperative interactions occur between catalytic sites. In the simplest case, a pair of sites

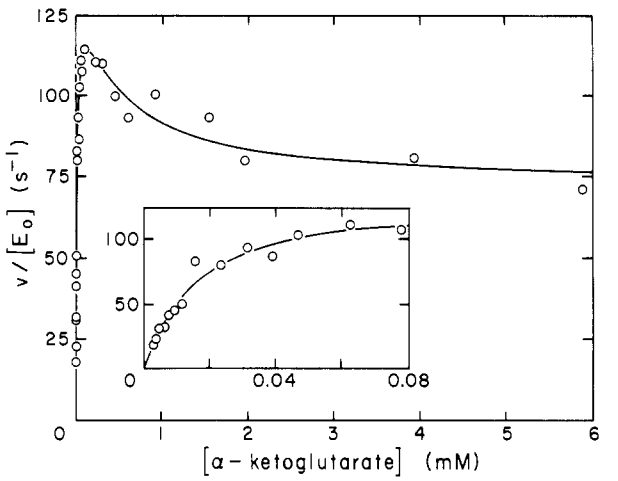

FIGURE 1: Plot of the initial steady-state velocity divided by the total enzyme concentration, $v/[E_0]$, vs. the concentration of α-ketoglutarate in 2 mM thiamin pyrophosphate, 2 mM $MgCl_2$, and 20 mM potassium phosphate (pH 7.0) at 4 °C. The enzyme complex concentration was 5.3 nM. The curve is the superimposable fit of the data to eq 7 and 8 with the parameters given in Table I.

is involved with the α-ketoglutarate binding constant and catalytic rate constant being different for the two sites:

$$E + 2S \underset{k_{-1}}{\overset{k_1}{\rightleftharpoons}} ES + S \underset{k_{-2}}{\overset{k_2}{\rightleftharpoons}} ES_2 \xrightarrow{\alpha k_{cat}} ES + P \xrightarrow{k_{cat}} E + 2P$$
$$\downarrow k_{cat} \qquad (6)$$
$$E + P + S$$

where E is the enzyme, S is the substrate, P is the product, and α is a constant less than 1. The initial steady-state velocity, v, for this mechanism is

$$\frac{v}{[E_0]} = k_{cat}\frac{[S] + \alpha[S]^2/K_2}{K_m + [S] + [S]^2/K_2} \qquad (7)$$

where $[E_0]$ is the total concentration of complex, K_m is the Michaelis constant $[(k_{cat} + k_{-1})/k_1]$, and $K_2 = (\alpha k_{cat} + k_{-2})/k_2$. A least-squares fit of the data to this mechanism gives the parameters in row 1 of Table I. In the second model, α-ketoglutarate can bind to a regulatory site that partially inactivates the enzyme when occupied. The initial steady-state velocity for this model is

$$\frac{v}{[E_0]} = k_{cat}\frac{[S] + \alpha[S]^2/K_2}{K_m + (1 + K_m/K_2)[S] + [S]^2/K_2} \qquad (8)$$

where k_{cat} is the turnover number for the complex in the absence of inhibition, α is the ratio of the turnover numbers of the fully inhibited to the fully active enzyme, K_m is the Michaelis constant, and K_2 is the dissociation constant for α-ketoglutarate binding at the regulatory site. The parameters obtained with a least-squares fit of the data to eq 8 are

presented in row 4 of Table I. The curves calculated with the appropriate parameters and eq 7 and 8 are included in Figure 1. Both mechanisms fit the data equally well, and the two curves are superimposable.

The dependence of the steady-state velocity of the E_1 component on the concentration of α-ketoglutarate at 4 °C was also investigated. Simple Michaelis–Menten kinetics with no substrate inhibition were observed, with k_{cat} = 39.3 (±0.9) s^{-1} and K_m = 10.8 (±1.9) μM.

Properties of the MalNEt-Treated Complex. Treatment of the α-ketoglutarate dehydrogenase complex with MalNEt inhibited the overall complex activity and the activity of the E_1 component. The extent of overall and E_1 activity loss agrees within 2–3% during the entire time course of the reaction of the enzyme with MalNEt. At high extents of inactivation (≥90% inhibition), a total of 14.2 mol of [^{14}C]MalNEt binds per mol of complex, and sodium dodecyl sulfate–polyacrylamide gel electrophoresis reveals that 12.5 mol is bound to E_1 subunits per mol of complex. The dependence of the initial steady-state velocity of the overall reaction on the concentration of α-ketoglutarate was examined at 4 °C in 20 mM potassium phosphate (pH 7.0) on enzyme with different extents of MalNEt modification. The activity was inhibited at high substrate concentrations, so the data were fit to the steady-state mechanisms described above. Table I includes the parameters for the fit of the data to eq 7 and 8 for two enzyme samples treated with MalNEt.

Succinylation Kinetics. The rate of succinylation of the α-ketoglutarate dehydrogenase complex by α-[5-^{14}C]ketoglutarate was studied with a quenched-flow apparatus. At times longer than a few seconds, a maximum of 13–16 mol of succinate binds per mol of complex. Varying the complex concentration from 16 nM to 1.6 μM, the α-ketoglutarate concentration from 4.7 μM to 2 mM, the thiamin pyrophosphate concentration from 0.25 to 2.5 mM, or the $MgCl_2$ concentration from 0.25 to 2.5 mM did not change the number of succinyl groups bound per mole of complex. In addition, the binding stoichiometries after the reaction was complete were unchanged when the reaction was run under nitrogen over the pH range 5.0–8.0, over the temperature range of 0–23 °C, or when the solutions were buffered with 20 mM potassium phosphate, 20 mM *N*-(2-hydroxyethyl)piperazine-*N*′-2-ethanesulfonic acid, or 20 mM 2-(*N*-morpholino)ethanesulfonic acid. In a few experiments, the α-ketoglutarate dehydrogenase complex was pretreated with 1.4 mM CoA, 1.4 mM dithiothreitol, and 1.4 mM NAD^+ in 20 mM potassium phosphate (pH 7.0) for 30 min on ice before being passed through two successive centrifuge columns. This procedure, which would remove any succinate groups previously bound to the enzyme and would oxidize all lipoic acids, did not alter the succinylation stoichiometry. The total extent of binding was also unchanged when the perchloric acid concentration used to quench the reaction was varied from 6% to 20%, or when the reaction mixture was quenched in 17 mM trichloroacetic acid and the labeled complex isolated on centrifuge columns. Quenching the reaction at neutral or alkaline pH resulted in a loss of label from the protein. All succinylation experiments described hereafter were performed at a complex concentration of 80 nM over an α-ketoglutarate concentration range of 4.7–74 μM in 2 mM thiamin pyrophosphate, 2 mM $MgCl_2$, and 20 mM potassium phosphate (pH 7.0) at 4 °C and were quenched in 13% perchloric acid. The substrate inhibition seen in the steady-state kinetic experiments is negligible over this range of α-ketoglutarate concentrations.

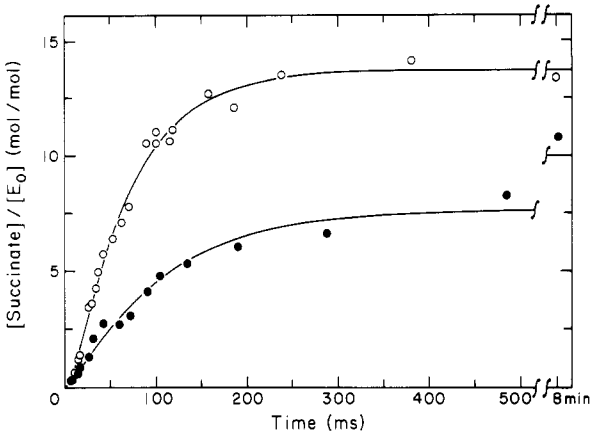

FIGURE 2: Time course of succinylation of the complex by α-ketoglutarate. The binding is expressed as the moles of succinate bound per mole of complex. The reaction was carried out with 80 nM complex that was unmodified (○) or treated with MalNEt to 38% E_1 activity remaining (●) in 22 μM α-ketoglutarate, 2 mM thiamin pyrophosphate, 2 mM $MgCl_2$, and 20 mM potassium phosphate (pH 7.0) at 4 °C. The curves were fit with the model described in the text (eq A1–A7).

A typical time course under these conditions is shown in Figure 2 (open circles). The succinylation is not a simple exponential process but is sigmoidal. The succinylation kinetics of complex in which varying amounts of the E_1 component have been inhibited with MalNEt have also been investigated. These experiments were performed under conditions identical with those used for the unmodified enzyme at a single α-ketoglutarate concentration of 22 μM. The amount of E_1 activity remaining was 32–60%. A typical time course for a MalNEt-treated complex is shown in Figure 2 (closed circles). As seen with the unmodified complex, the time course is sigmoidal, but the stoichiometry plateaus at a lower level at short times (<1 s). When the reaction is allowed to proceed for several minutes, the binding stoichiometry increases slowly to 11–15 mol per mol of complex and closely approaches the limiting value observed for the unmodified complex.

The kinetic data were fit to two limiting models. Both models assume that the initial binding of α-ketoglutarate to the E_1 subunit is reversible and fast compared to the decarboxylation. Furthermore, the substrate concentration is much greater than the concentration of enzyme catalytic sites so that the reaction can be assumed to be pseudo first order with respect to the enzyme. In the first model, the succinylation occurs via two sequential steps:

$$E \xrightarrow{k_1} SE \xrightarrow{k_2} ES \qquad (9)$$

where E is the enzyme complex, SE is an intermediate unstable in the acid quench conditions, ES is the acid quench stable succinylated complex, and k_1 is a function of the substrate concentration. The observed time courses are inconsistent with a model in which both SE and ES are acid-stable species. For this mechanism

$$[ES]/[E_0] = A[1 - (k_2e^{-k_1t} - k_1e^{-k_2t})/(k_2 - k_1)] \qquad (10)$$

where $[E_0]$ is the total amount of enzyme and A is the succinylation stoichiometry (mole per mole) at infinite time. An additional exponential term (Be^{-k_3t}) with a first-order rate constant of 1.3–6.1 min^{-1} is included in the cases when the succinylation of the MalNEt-treated complex is studied to account for the additional slower process. The data were fit to eq 10, and Figure 3 shows the dependence of the succinylation stoichiometry, A, and the rate constants, k_1 and k_2, on α-ketoglutarate concentration. The data for the unmodified

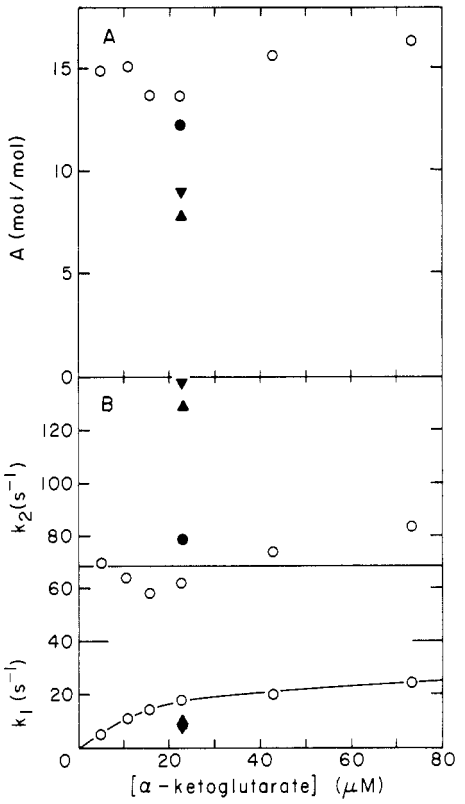

FIGURE 3: Plots of the moles of bound succinate per mole of complex at infinite time, A (A), and of the rate constants, k_1 and k_2 (B), for succinylation of the complex by α-ketoglutarate vs. the concentration of α-ketoglutarate, when the time courses were fit to eq 10. The reaction was carried out with unmodified complex (○) or with complex treated with MalNEt to remaining E_1 activities of 60% (●), 38% (▲), or 32% (▼) under the same experimental conditions as described in the legend to Figure 1. The average values of A and k_2 for the unmodified complex were 14.8 mol/mol and 69 s^{-1}, respectively. The curve drawn through the values of k_1 was calculated with eq 11 and the best-fit parameters $k_1' = 30.8$ s^{-1} and $K_D = 17.9$ μM.

complex are displayed as open circles, and the data for the MalNEt-treated complex are shown as filled symbols. The limiting stoichiometry for the unmodified complex is constant within experimental error and has an average value of 14.8 (±1.1) mol per mol of complex for six time courses, while the values of A for the modified complex are reduced. Although k_1 and k_2 are symmetric in eq 10, the two rate constants can be distinguished by their dependence on the substrate concentration. For the unmodified enzyme, the smaller rate constant increases with α-ketoglutarate concentration and approaches a limiting value. This observation is consistent with the assignment of this rate constant to k_1 and the assumption of the rapid formation of an enzyme–substrate complex followed by an irreversible rate-limiting step. For this mechanism

$$k_1 = k_1'/(1 + K_D/[S]) \qquad (11)$$

where [S] is the substrate concentration, K_D is the dissociation constant for the initial enzyme–substrate complex, and k_1' is the limiting rate constant at high substrate concentrations. Figure 3B shows the fit of the data to this mechanism, with $k_1' = 30.8$ (±2.2) s^{-1} and $K_D = 17.9$ (±3.2) μM. The larger rate constant is independent of substrate concentration within experimental error and has an average value of 69 (±9) s^{-1}. This observation is consistent with assignment of this rate constant to k_2. The rate constants for the MalNEt-treated complex do not agree well with those for the unmodified enzyme. When k_2 for the modified complex is fixed at 69 s^{-1} in the fitting procedure, k_1 remains considerably lower than the value for the unmodified enzyme (Figure 3B). These observations are inconsistent with the steady-state kinetic data, which suggests that modification with MalNEt completely inhibits an enzyme molecule. If this is the case, the observed rate constants would not be expected to change. In addition, the number of E_1 subunits that would remain active after MalNEt treatment is significantly lower than the number of groups succinylated within a few seconds, indicating that an E_1 subunit can succinylate more than a single lipoic acid. For example, enzyme that has only 4.55 active E_1 subunits (on the basis of 37.9% residual E_1 activity) can succinylate 7.81 lipoic acids.

The following model is consistent with the above observations. Two classes of E_1 subunits are assumed. The first class of E_1 subunits succinylates a single lipoic acid per E_1 by the same two-step mechanism as described previously, with rate constants k_1 and k_2 (eq 9). The second class of E_1 subunits succinylates two lipoic acids per E_1 by two successive two-step reactions, with the same rate constants k_1 and k_2 for the elementary steps. The distribution of E_1 subunits between the two classes is chosen to match the experimentally determined limiting number of succinylated lipoic acids with the available number of active E_1 subunits. The stoichiometry of active E_1 subunits is 12 per complex for unmodified enzyme, or is assumed to be proportional to the residual E_1 activity when the complex is treated with MalNEt. Details of this model are described in the Appendix. As noted above, an additional slow succinylation process (rate constant 2.7–9.1 min^{-1}) is observed when the complex is treated with MalNEt. The curves in Figure 2 are calculated with this model. The limiting stoichiometry and the rate constants obtained with this model are shown in Figure 4 at various α-ketoglutarate concentrations. The data for the fully active complex are shown as open circles, and the data for the MalNEt-inhibited enzyme are presented as filled symbols. The limiting stoichiometry for the unmodified complex is constant and has an average value of 14.3 ± 1.0 mol per mol of complex for the six time courses, and is reduced for the modified complex. Again, the substrate dependence of k_1 is consistent with the rapid formation of an enzyme–substrate complex followed by an irreversible first-order process. The fit of the data for the unmodified enzyme to eq 11 gives $k_1' = 49$ (±3) s^{-1} and $K_D = 34.3$ (±3.7) μM. The rate constant k_2 is constant within experimental error and has an average value of 89 (±16) s^{-1}. Thus, this mechanism is consistent with the kinetics of succinylation for the MalNEt-modified and the unmodified enzyme by α-ketoglutarate.

Desuccinylation Kinetics. The desuccinylation reaction was studied by reacting CoA with enzyme complex that had been previously labeled with α-[5-^{14}C]ketoglutarate and by monitoring either the label remaining on the complex or the [4-^{14}C]succinyl-CoA produced. The reaction was carried out at 4 °C with 80 nM complex, 21–23 μM α-[5-^{14}C]ketoglutarate, 2 mM thiamin pyrophosphate, 2 mM $MgCl_2$, 1 mM dithiothreitol, and 38 or 270 μM CoA in 20 mM potassium phosphate (pH 7.0). The experiments reveal that about 74% of the succinyl groups are removed from the enzyme by CoA under these conditions and that this reaction is completed during the shortest time accessible (7 ms). Therefore, the first-order rate constant for the desuccinylation reaction is greater than 200 s^{-1}. At reaction times of 7 ms to several minutes, 3.5 and 3.3 mol of succinate remain bound per mol of complex at 38 and 270 μM CoA, respectively. At reaction times of 7 ms to 1 s, 9.3 and 9.7 mol of [4-^{14}C]succinyl-CoA are produced per mol of complex at 38 and 270 μM CoA, respectively. When the reaction is allowed to proceed for several minutes, greater amounts of [4-^{14}C]succinyl-CoA are

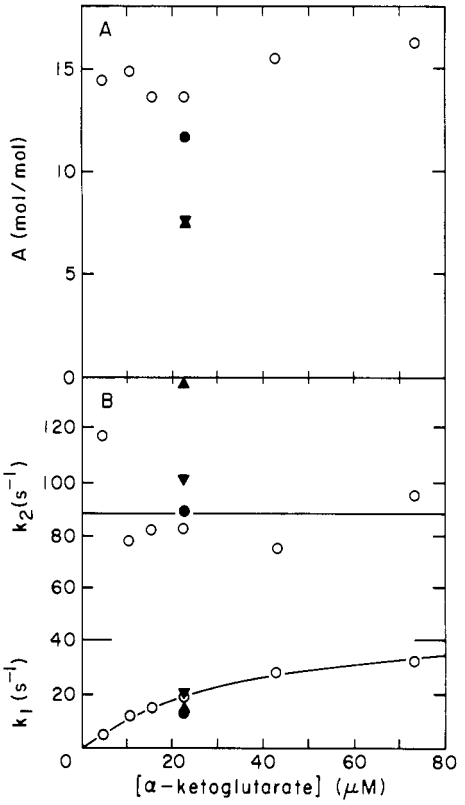

FIGURE 4: Plots of the moles of bound succinate per mole of enzyme at infinite time, A (A), and of the rate constants, k_1 and k_2 (B), for succinylation of the complex by α-ketoglutarate vs. the concentration of α-ketoglutarate, when the time courses were fit to eq A2, A4–A6, and 11. The reaction was carried out with unmodified complex (○) or with complex treated with MalNEt to remaining E_1 activities of 60% (●), 38% (▲), or 32% (▼) under the same experimental conditions as described in the legend to Figure 1. The average values of A and k_2 for the unmodified complex were 14.3 mol/mol and 89 s^{-1}, respectively. The curve drawn through the values of k_1 was calculated with eq 11 and the best-fit parameters $k_1' = 49$ s^{-1} and $K_D = 34.3$ μM.

produced (~20 mol/mol after 8 min with 270 μM CoA). This is probably due to air oxidation of lipoic acid which allows the enzyme reaction to cycle catalytically, while the amount of label bound to the complex remains constant. If 300 μM CoA is added to the succinylated enzyme 4–5 s after succinylation (without isolation of the succinylated enzyme), less than 5% of the radioactivity remains on the isolated enzyme. This indicates that the tightly bound succinyl groups are generated during the isolation of the succinylated enzyme in a relatively slow reaction.

Discussion

The steady-state kinetic data obtained with the α-ketoglutarate dehydrogenase complex indicate that the reaction of the complex is regulated by α-ketoglutarate. The data are consistent with regulation either by cooperative interactions between catalytic sites or by an α-ketoglutarate inhibitory site. These mechanisms cannot be distinguished, but the pairwise cooperativity between catalytic sites is consistent with the known dimeric structure of E_1, and the turnover number of the enzyme in the inhibited state is approximately half the turnover number of the most active state. The activity of E_1, as measured by the ferricyanide assay, is not inhibited at high concentrations of α-ketoglutarate; however, the rate-determining step is different from that for the overall reaction since the turnover numbers for the two reactions are different. Since the inhibition occurs at higher concentrations of α-ketoglutarate than are physiologically relevant, a more detailed study of the inhibition was not pursued.

The multienzyme complex can be inhibited by specifically inactivating the E_1 component with MalNEt. The limiting value of 12.5 mol of MalNEt binding to the E_1 component per mol of complex closely matches the subunit stoichiometry of 12 E_1 subunits per complex, suggesting that a single sulfhydryl group on each E_1 is labeled. This labeling is distinct from the modification of the lipoic acids on E_2 by maleimides in the presence of α-ketoglutarate (Angelides & Hammes, 1979; Waskiewicz & Hammes, 1982). The steady-state kinetic data for the MalNEt-treated complex show the same substrate inhibition seen for the unmodified enzyme, although the dissociation constant for α-ketoglutarate binding to the second or inhibitory site is greatly reduced (Table I). The inactivation by MalNEt is accompanied by a lowering of k_{cat} proportional to the extent of inactivation, while the Michaelis constant is unchanged within experimental error. These observations are consistent with MalNEt acting as an irreversible inhibitor of the E_1 subunit. The parallel inhibition of the E_1 enzyme and overall activity indicates that the oxidation of α-ketoglutarate is inhibited: i.e., the inhibition occurs *before* succinylation of the complex. In the case of another E_1 inhibitor, thiamin thiazolone pyrophosphate, the activity loss was proportional to the amount of bound inhibitor (Angelides & Hammes, 1979). Therefore, in interpretation of the transient kinetics, the number of active E_1 subunits is assumed to be 12 for the unmodified enzyme and proportional to the remaining E_1 activity for the MalNEt-modified enzyme.

Earlier studies have shown that each α-ketoglutarate dehydrogenase complex contains 24 lipoic acids (Pettit et al., 1973; Collins & Reed, 1977; Angelides & Hammes, 1979; White et al., 1980). The work presented here indicates that under a wide variety of experimental conditions only 13–16 lipoic acids per complex are succinylated by α-ketoglutarate. The hydroxybutyrylthiamin pyrophosphate is not stable under the acid quench conditions (Speckherd et al., 1977; Akiyama & Hammes, 1980). The succinylation stoichiometry reached within 1 s is reduced further when some of the E_1 subunits are inactivated with MalNEt, although the incorporation slowly approaches the values found for the unmodified complex when the succinylation reaction proceeds for several minutes. This slower process is unlikely to be of catalytic significance. These results indicate a limited accessibility of the lipoic acids to the E_1 components on a catalytic time scale. Previously, Pettit et al. (1973) found that 12 of the 24 lipoic acids could be succinylated by α-ketoglutarate, while Collins & Reed (1977) reported all 24 groups could be succinylated.

The time course of succinylation of the enzyme by α-ketoglutarate is sigmoidal and indicates that the succinylation occurs via a minimum of two sequential steps. The data can be fit by a simple two-step mechanism (eq 9 and 10), but the rate constants associated with the initial reaction of the E_1-inhibited complex (k_1) are smaller than those for the unmodified enzyme. The average number of lipoic acids succinylated per active E_1 is greater for the modified enzyme than for the unmodified enzyme. This observation is consistent with the reduction in the rate constants being due to the greater number of successive succinylations required by the active E_1 subunits of the modified complex. The data also were fit to a second mechanism allowing for some of the E_1 subunits to succinylate two lipoic acids successively, each by a two-step process (eq A1–A7). For this model, the rate constants associated with the modified and unmodified complex agree closely and have values of about 49 s^{-1} (k_1) and 89 s^{-1} (k_2) for the first and second steps of the succinylation, respectively.

The first step in the reaction could be the decarboxylation on E_1 and the second step the transfer to a lipoyl group on E_2.

Desuccinylation of the complex by CoA is rapid and has a rate constant greater than 200 s^{-1}. Only 9–10 succinyl groups are removed by CoA, however, while 3.3–3.5 succinates appear to irreversibly bind to the complex. These tightly bound succinyl groups cannot be the result of the reverse reaction of eq 3 since the number of bound groups is independent of the CoA concentration. The irreversibly bound succinyl groups are due to a relatively slow transfer from lipoic acid to other groups on the enzyme during isolation of the succinylated enzyme and are unlikely to be of catalytic significance.

The data presented suggest a picture of how the multiple subunit structure of the α-ketoglutarate dehydrogenase complex mediates the transfer of intermediates. The low succinylation stoichiometry, reduced further when a number of E_1 subunits are inactivated, can be attributed to a limited accessibility between E_1 subunits and the lipoic acids on E_2. Wagenknect et al. (1983) have examined the structure of reconstituted subcomplexes and found that the E_1 dimers bind to 6 out of 24 apparently identical sites on the E_2 core at random. This heterogeneous structure of the complex implies that not all lipoic acids may be accessible to an E_1 subunit. The average number of lipoic acids succinylated per active E_1 approaches two as increasing amounts of E_1 are inactivated, suggesting that each E_1 is available to no more than two lipoic acids. The data are consistent with a model requiring two classes of E_1 subunits on each complex, one class succinylating a single lipoic acid per E_1 and a second class succinylating two lipoic acids per E_1. Other models clearly are consistent with the data. For example, individual E_1 subunits may succinylate two or more lipoic acids, with the lower stoichiometry resulting from competition among E_1 components for the available lipoic acids. However, insufficient information is available to evaluate such complex models. A computer modeling of the reaction cycle suggests that each E_1 molecule services two lipoic acids (Hackert et al., 1983). Succinyl group transfers between lipoic acids probably do not contribute significantly to the reaction cycle because of the limited succinylation stoichiometry and the high degree of symmetry of the transsuccinylase core. Angelides & Hammes (1979) have demonstrated that the enzyme complex is almost fully active with only eight active E_3 components per complex, implying that electron transfers between lipoic acids may occur.

The mechanism of action of the α-ketoglutarate dehydrogenase complex differs from the structurally and mechanistically similar pyruvate dehydrogenase complex from *E. coli*. All 48 lipoic acids can be acetylated by pyruvate (Collins & Reed, 1977; Akiyama & Hammes, 1980), and transfers of acetyl groups between lipoic acids are likely to occur (Collins & Reed, 1977; Danson et al., 1978; Angelides & Hammes, 1978; Ambrose-Griffin et al., 1980). The incorporation of pyruvate can be analyzed in terms of a fast process (rate constant 45–60 s^{-1}) plus a slower process not of catalytic significance (Akiyama & Hammes, 1980). The deacetylation by CoA is very fast, and irreversible binding of acetyl groups occurs, similar to the α-ketoglutarate dehydrogenase complex.

The turnover number for the complex cannot be readily compared with the rate constants for the elementary reactions because each E_1 may service more than one lipoic acid and not all lipoic acids may be capable of participating in a catalytic cycle. If 12 parallel reaction pathways per complex are assumed [on the basis of a subunit stoichiometry of 12:24:12 (E_1:E_2:E_3)], the turnover number for the complex of 140–150 s^{-1} can be regarded as 12–13 s^{-1} per reaction cycle. Comparing this value with rate constants for the two-step succinylation of 49 and 89 s^{-1} suggests that an additional rate-limiting step of about 20 s^{-1} may occur in the reaction cycle or that the complex contains fewer than 12 parallel reaction paths. (This assumes $k_{cat} \approx [\sum_i (1/k_i)]^{-1}$ where the k_i values are the consecutive first-order rate constants characterizing the catalytic process on the enzyme.) The difference between the enzyme–substrate dissociation constant of 34 μM based on the α-ketoglutarate concentration dependence of k_1 and the Michaelis constant of 19–20 μM can be almost entirely accounted for by the second succinylation step (k_2). This observation suggests that no remaining slow steps occur in the reaction cycle and that fewer than 12 parallel reaction paths occur within the complex.

Appendix

Model for Succinylation of the α-Ketoglutarate Dehydrogenase Complex. This model is based on the assumption that two classes of E_1 subunits exist: one class consists of subunits that succinylate a single lipoic acid per E_1, and another class consists of subunits that succinylate two lipoic acids per E_1. Each succinylation is assumed to occur via an initial decarboxylation and subsequent transfer to the lipoic acid; the rate constants associated with these two elementary steps are the same for the two classes of E_1 subunits. Since the substrate concentration is in excess, the mechanism for succinylation by the first class of E_1 subunits can be written as

$$E \xrightarrow{k_1} SE \xrightarrow{k_2} ES \qquad \text{(A1)}$$

where E is the enzyme, SE is an intermediate unstable to acid quench conditions, ES is the succinylated lipoic acid form of the enzyme, and k_1 is a function of the substrate concentration. For this mechanism

$$y_1 = N_1[1 - (k_2 e^{-k_1 t} - k_1 e^{-k_2 t})/(k_2 - k_1)] \qquad \text{(A2)}$$

where y_1 is the number of succinylated lipoic acids of this class per enzyme complex and N_1 is the number of E_1 subunits per complex that succinylate a single lipoic acid each. The second class of E_1 subunits succinylates two lipoic acids successively with the same elementary rate constants:

$$E \xrightarrow{k_1} SE \xrightarrow{k_2} ES \xrightarrow{k_1} SES \xrightarrow{k_2} ES_2 \qquad \text{(A3)}$$

where S to the left of E indicates an acid-unstable intermediate and S to the right of E indicates the succinylated lipoic acid, analogous to above. For this class

$$\frac{[ES]}{[E_0]} = \frac{N_2 k_1 k_2}{k_2 - k_1}\left(\frac{e^{-k_2 t} - e^{-k_1 t}}{k_2 - k_1} + t e^{-k_1 t}\right) \qquad \text{(A4)}$$

$$\frac{[SES]}{[E_0]} = \frac{N_2 k_1^2 k_2}{(k_2 - k_1)^2}\left(\frac{2e^{-k_2 t} - 2e^{-k_1 t}}{k_2 - k_1} + t e^{-k_1 t} + t e^{-k_2 t}\right) \qquad \text{(A5)}$$

$$\frac{[ES_2]}{[E_0]} = N_2\left\{1 - \left(\frac{k_1 k_2}{k_2 - k_1}\right)^2\left[\left(\frac{1}{k_1^2} - \frac{2}{k_1(k_2 - k_1)}\right)e^{-k_1 t} + \left(\frac{1}{k_2^2} + \frac{2}{k_2(k_2 - k_1)}\right)e^{-k_2 t} + \frac{t e^{-k_1 t}}{k_1} + \frac{t e^{-k_2 t}}{k_2}\right]\right\} \qquad \text{(A6)}$$

where $[E_0]$ is the total amount of enzyme complex and N_2 is the number of E_1 subunits per complex that succinylate two lipoic acids each. The number of succinylated lipoic acids of the second class per complex, y_2, is given by

$$y_2 = [ES]/[E_0] + [SES]/[E_0] + 2[ES_2]/[E_0] \qquad \text{(A7)}$$

where the terms are defined by eq A4–A6. The total succinylation stoichiometry is the sum of the contributions from the two parallel paths, $y_1 + y_2$.

The total number of active E_1 subunits per complex, $N_1 + N_2$, is assumed to be 12 for the unmodified complex, or to be reduced proportionally when the E_1 activity is reduced with MalNEt. The fitting procedure used distributes these subunits between the two classes described above to match the observed limiting succinylation stoichiometry, while simultaneously fitting k_1 and k_2. For example, this mechanism would account for the succinylation of 15 lipoic acids by 12 E_1 subunits by requiring that 9 E_1 subunits succinylate 1 lipoic acid per E_1 and 3 E_1 subunits succinylate 2 lipoic acids per E_1. After the values of N_1 and N_2 were fixed, the time courses were fit directly to eq A2 and A7.

Registry No. MalNEt, 128-53-0; E_1, 37205-42-8; α-ketoglutarate dehydrogenase, 9031-02-1; α-ketoglutaric acid, 328-50-7.

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Isotope, Pulse–Chase, Stopped-Flow, and Rapid Quench Studies on the Kinetic Mechanism of Bovine Dihydropteridine Reductase†

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ABSTRACT: The kinetics of the reduction of quinonoid 2-amino-4-hydroxy-6,7-dimethyldihydropteridine ($DMPH_2$) catalyzed by bovine liver dihydropteridine reductase were examined with NADH, (*S*)-NADD, (*S*)-NADT, and [^{3}H]-NADH as substrates. No significant deuterium isotope effect was observed on either K_m or V_m, indicating that hydrogen transfer is not a major rate-limiting step of the reaction. Tritium from (*S*)-NADT is transferred to an exchangeable position of the pteridine product without significant isotopic discrimination. The ratio of tritium released into solvent to NAD^+ produced is approximately 1.0 in the steady state as well as in the first enzyme turnover as determined by pulse–chase experiments. Pulse–chase methods also showed that the binary complex E·NADH is fully functional and can be completely converted to products prior to NADH dissociation in the presence of saturating $DMPH_2$. The concentration of $DMPH_2$ giving half-maximal trapping of E·NADH is identical with its K_m as determined by steady-state kinetics. Stopped-flow kinetic measurements gave no evidence for a burst of NADH utilization. This was further demonstrated by rapid quench experiments which demonstrated a pre-steady-state rate nearly identical with that of the steady state. The above results are consistent with nonequilibrium ordered binding of substrates and with a rate-limiting isomerization in the ternary complex which precedes hydrogen transfer.

Dihydropteridine reductase (DHPR)[1] (EC 1.6.99.7) reduces the quinonoid dihydropteridine formed during hydroxylation of aromatic compounds (Kaufman & Fisher, 1974). Kinetic analysis of the bovine liver enzyme (Chauvin et al., 1979; Asknes & Ljones, 1980) using quinonoid 6,7-dimethyldihydropterin ($DMPH_2$) as a substrate, the observation that one NADH per monomer is tightly bound (Hasegawa, 1977; Webber & Whiteley, 1978; Chauvin et al., 1979), and results with affinity chromatography (Korri et al., 1977; Chauvin et

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[1] Abbreviations: DHPR, dihydropteridine reductase; $DMPH_2$, quinonoid 2-amino-4-hydroxy-6,7-dimethyldihydropteridine; $DMPH_4$, 2-amino-4-hydroxy-6,7-dimethyltetrahydropteridine; NAD^+, nicotinamide adenine dinucleotide; [^{3}H]NAD^+, NAD^+ with general tritium label in the adenosine ring; NADH, reduced nicotinamide adenine dinucleotide; NADD, 4-deuterio-NADH; NADT, 4-tritio-NADH; [^{3}H]NADH, NADH with general tritium label in adenosine ring; NAD* or NADH*, NAD^+ or NADH radioactively labeled; Tris-HCl, tris(hydroxymethyl)aminomethane hydrochloride.

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