α-KETOGLUTARATE IN BIOLOGICAL HYDROXYLATIONS

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1. Introduction

Oxygen-requiring hydroxylations have been classified as monooxygenase or mixed-function oxidase reactions, and usually require a reducing cofactor such as a tetrahydropteridine or ascorbate [1,2]. In some cases the electrons for the reduction of the oxygen molecule are donated by a part of the substrate molecule other than that which is being hydroxylated (see ref. [3]). Recently, several hydroxylations in which ascorbate has been believed to be the reducing cofactor have been found to require α-ketoglutarate, viz. protocollagen hydroxylase (collagen proline hydroxylase) [4,5], collagen lysine hydroxylase [6,7], γ-butyrobetaine hydroxylase of animal or bacterial origin [8,9] and thymine-7-hydroxylase [10]. A requirement for divalent iron has been demonstrated for these hydroxylases. It has been suggested [11] that α-ketoglutarate acts as an allosteric effector, but recent studies with γ-butyrobetaine hydroxylase have demonstrated a stoichiometric relationship between the formation of hydroxylated product – carnitine – and the disappearance of α-ketoglutarate [12].

In these studies succinate was identified as a degradation product of α-ketoglutarate. Subsequently, similar results were reported with collagen proline hydroxylase [13]. A function of α-ketoglutarate as an allosteric effector is therefore less probable.

One may envisage different mechanisms for the formation of succinate. (i) Succinic semialdehyde is an intermediate. α-Ketoglutarate is required by the enzyme either for the generation of succinic semialdehyde which serves as reductant or for the binding of ferrous ions, degradation to succinic semialdehyde being a side reaction during the hydroxylation. In the latter case succinic semialdehyde is non-specifically oxidized to succinate. (ii) Direct formation of succinate from α-ketoglutarate in a concerted reaction concomitantly with the hydroxylation of the substrate. In the present communication we present evidence for alternative (ii) and suggest a reaction mechanism for α-ketoglutarate-requiring hydroxylations.

2. Experimental procedures

Partially purified γ-butyrobetaine hydroxylase was prepared from *Pseudomonas sp.* AK 1 [14] by chromatography on hydroxylapatite and on DEAE cellulose as will be described in detail elsewhere. The incubation system for hydroxylation of γ-butyrobetaine to carnitine was: sodium ascorbate (10 μmoles), ferrous sulfate (0.4 μmole), catalase (1 mg), nicotinamide (40 μmoles), potassium chloride (20 μmoles), and potassium phosphate buffer at pH 7.0 (25 μmoles) in a final volume of 0.7 ml. γ-Butyrobetaine or [*methyl*-^{14}C] γ-butyrobetaine [15] (2 μmoles, 0.5 μC); α-ketoglutarate or [5-^{14}C] α-ketoglutarate (The Radiochemical Centre, Amersham, Bucks., England) or [1-^{14}C] α-ketoglutarate, *as indicated*; succinic semialdehyde, *as indicated.* The incubations were carried out at 37° for 45 min. The conversion to carnitine was determined in incubations with [methyl-^{14}C] γ-butyrobetaine by ion exchange chromatography of the reaction mixture [16]. The formation of $^{14}CO_2$ was determined in incubations with [1-^{14}C] α-ketoglutarate, obtained by treatment of [1-^{14}C] glutamic acid (The Radiochemical Centre, Amersham, Bucks., England) with oxaloacetate and L-aspartate: 2-oxoglutarate aminotransferase (EC 2.6.1.1) (Koch-Light Laboratories Ltd., Colnbrook, Bucks, England). These incubations were carried out in a Conway cham-

ber containing 0.2 ml of 1 M Hyamine solution in the center well. Succinic semialdehyde was prepared according to Carrière as modified by Albers [17,18]. Succinic semialdehyde was determined by the modification of the methods of Bessman [19], and Prescott and Waelsch [20]. Paper chromatography of the dinitrophenylhydrazones of succinic semialdehyde and α-ketoglutarate was performed in butanol : 95% aqueous ethanol : 0.5 M NH_4OH, 130:20:50 [21]. The paper chromatograms were scanned for radioactivity in a radiochromatogram scanner (Labor. Prof. Dr. Berthold, Wildbad, West Germany). Other isotope determinations were done in a liquid scintillation spectrometer (Packard Instrument Company, Inc., Downers Grove, Ill., USA) with an efficiency for ^{14}C of about 25%.

3. Results and discussion

It is apparent from table 1 that succinic semialdehyde cannot replace α-ketoglutarate as a cofactor for γ-butyrobetaine hydroxylase and from table 2 that there is no decrease in the amount of $^{14}CO_2$ formed from [1-^{14}C] α-ketoglutarate when succinic semialdehyde is added to the system. These data support the contention that degradation of α-ketoglutarate is obligatory in the hydroxylation and rule out the possibility that the function of α-ketoglutarate is to furnish succinic semialdehyde. The data in table 3 exclude a nonspecific formation of succinate from succinic semialdehyde and therefore suggest that also the formation of succinate occurs as a consequence of the hydroxylation reaction. When the hydroxylation was carried out in the presence of [5-^{14}C] α-ketoglutarate and an excess of succinic semialdehyde there was no incorporation of isotope in the reisolated succinic semialdehyde. This speaks in favor of a concerted reaction mechanism (alternative (ii)) in which free succinic semialdehyde does not occur as an intermediate. We would therefore suggest the following reaction mechanism for the hydroxylation (scheme 1).

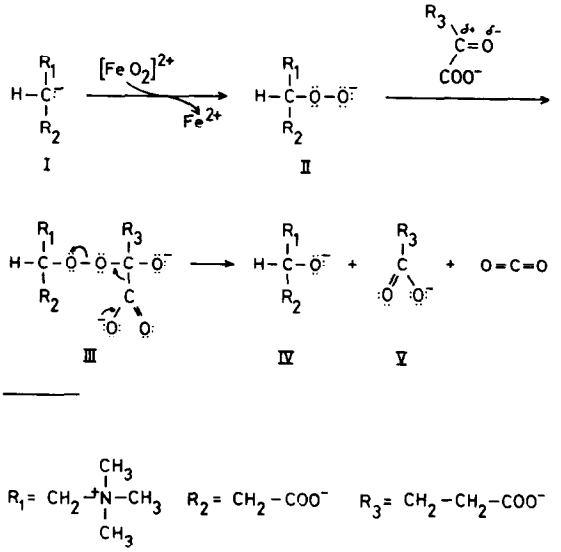
Table 1
Formation of carnitine in the presence of succinic semialdehyde and α-ketoglutarate. Partially purified enzyme was incubated with 2 μmoles of [methyl-$^{14}CH_3$] γ-butyrobetaine. Cofactors other than succinic semialdehyde and α-ketoglutarate were those given in experimental procedures.

Cofactor		Carnitine formed (μmoles)
Succinic semialdehyde (μmoles)	α-ketoglutarate (μmoles)	
0.0	0.0	<0.02
2.7	0.0	<0.02
0.0	1.9	1.45
2.7	1.9	1.32

Table 2
Formation of $^{14}CO_2$ from [1-^{14}C] α-ketoglutarate with and without succinic semialdehyde. To all incubations was added 0.002 μmole of [1-^{14}C] α-ketoglutarate (~0.10 μC). In the absence of enzyme <300 cpm were recovered as $^{14}CO_2$.

Addition (μmoles)		$^{14}CO_2$ (cpm)
		46,000
Succinic semialdehyde	(0.85)	43,000
Succinic semialdehyde	(3.4)	44,000
α-ketoglutarate	(2.0)	10,000

At the present time, we believe the following sequence of events to take place. The rate-limiting step is apparently the dissociation of the proton from the C-3 carbon atom of γ-butyrobetaine to form an anion (I) [15]. An oxygen molecule, its reactivity being enhanced as a result of complexing with a ferrous ion, attacks anion (I), forming a complex of the following



Scheme 1.

Table 3
Non-disappearance of succinic semialdehyde. The complete system was incubated with α-ketoglutarate and succinic semialdehyde.

α-Ketoglutarate (μmoles)	Succinic semialdehyde before incubation (μmoles)	Succinic semialdehyde after incubation (μmoles)	Carnitine formed (μmoles)
2.0	10.5	10.5	0.6
2.0	0.8	0.8	0.9
0.1	9.0	9.0	0.05

type

$$[Fe...O_2{-}CH(R_1)R_2]^+ .$$

Simultaneously with the dissociation of the ferrous ion from this complex, the peroxide anion (II) thus formed makes a nucleophilic attack on the C-2 carbon atom of α-ketoglutarate. The resulting peroxide (III) then disintegrates to the carnitine anion (IV), succinate (V) and carbon dioxide. In this reaction mechanism γ-butyrobetaine and α-ketoglutarate are cosubstrates for the "hydroxylase" which might formally be regarded as a dioxygenase *. There is no need for an exogenous reducing cofactor. A similar mechanism may be formulated for the oxygenation of *p*-hydroxyphenylpyruvate to homogenisate. In that reaction the hydroxylation of the benzene ring is coupled to the oxidative degradation of the pyruvate side chain [23]. The initial events may be the same also in the formation of acetate from lactate, catalyzed by lactic oxidative decarboxylase [24]. Ascorbate stimulates the hydroxylation of γ-butyrobetaine [15,25] but may be replaced by other reductants, most of which, however, are less active than ascorbate (unpublished results). Several reductants including ascorbate stimulate the formation of homogenisate from *p*-hydroxyphenylpyruvate possibly by stabilizing the enzyme [26]. The function of the reducing agents in the hydroxylations discussed here might be to keep ionic iron in the divalent form and possibly to protect SH groups from oxidation.

* According to the rules for the naming of enzymes [22] the systematic name of γ-butyrobetaine hydroxylase would be 4-trimethylaminobutyrate, 2-oxoglutarate: oxygen oxidoreductase (4-trimethylaminobutyrate-3-hydroxylating).

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