FERREDOXIN IS THE COENZYME OF α-KETOACID OXIDOREDUCTASES IN *HALOBACTERIUM HALOBIUM*

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1. Introduction

Halobacterium halobium cells contain a 2Fe-2S-ferredoxin which resembles more the plant-type ferredoxins than 2Fe-2S-ferredoxins from other bacteria [1–3]. Halobacteria are respiratory organisms [4] which are also capable of bacteriorhodopsin-dependent photophosphorylation [5,6]. The existence of the citric acid cycle has been established; however, the oxidation of α-ketoglutarate could only be demonstrated by the use of an artificial electron acceptor (iodonitrophenol tetrazolium chloride) [7], but not by reduction of pyridine nucleotides. Other enzymes oxidizing α-ketoacids are not known in Halobacteria.

Fermentative anaerobes [8,9], photosynthetic bacteria [8,10] and some blue-green algae [11,12] are able to catalyze α-ketoacid oxidation (or synthesis) by their α-ketoacid-ferredoxin oxidoreductases. This paper presents evidence that in *Halobacterium halobium* ferredoxin functions as the physiological electron acceptor in the enzymic oxidation of α-ketoglutarate, pyruvate and α-ketobutyrate.

2. Materials and methods

2.1. *Biological materials*

Halobacterium halobium NRL cells were cultured in 2 litre Erlenmeyer flasks (40°C, 180 rev./min, 65 h) containing 1 litre of the peptone medium described in [13]. The cells were suspended in 2.5 ml 3.0 M KCl in 50 mM Tris–Cl, pH 8.0, per gram cell paste, and DNAase (300 U/mg, Roth KG, Karlsruhe) was added (0.2 mg/ml). Cell rupture occurred upon freezing in liquid nitrogen and the lysate was allowed to stand at room temperature for 2 h. During the following operations the temperature was kept at 5°C. The bacterial extract obtained by centrifugation at 40 000 × *g* for 3 h contained 35–40 mg/ml protein. It was dialyzed against 3 M KCl buffered with 50 mM Tris–HCl, pH 8, in order to remove endogenous substrates. Alternatively the extract (20 mg/ml) was chromatographed on Ultrogel AcA 22 (LKB) and eluted with 3.0 M KCl in 50 mM Tris–Cl, pH 8.0.

Halobacterium halobium ferredoxin was prepared as described in [2] but without 2-mercaptoethanol.

2.2. *Assay methods*

All assays were performed at 25°C in the presence of 3.0 M KCl.

2.2.1. Cytochrome *c* reduction assay

A cuvette with 10 mm light path contained the following: 3.0 M KCl, 50 mM Tris–HCl, pH 8.0, 1 mM Na_4EDTA, 20 μM *H. halobium* ferredoxin, 50 μM horse heart cytochrome *c* (grade II-A from Sigma), 1 mM of either sodium pyruvate or sodium α-ketobutyrate or 5 mM sodium α-ketoglutarate and enzyme extract (0.1–1 mg protein/ml). The reaction was started by the addition of 5 μl 10 mM coenzyme A to 1 ml reaction mixture. Reduction of 1 nmol cytochrome *c* corresponds to an $A_{550\ nm}$ increase by 0.021 [14].

2.2.2. Assay of hydroxamic acid formation

The reaction mixture contained 3.0 M KCl, 50 mM Tris–HCl, pH 8.0, 1 mM Na_4EDTA, 2 mM dithioerythrol, 40 μM *H. halobium* ferredoxin, 10 mM

α-ketoacid and enzyme extract (5–15 mg protein/ml). The reaction was started by the addition of coenzyme A (2.5 mM final concentration). To ensure reoxidation of ferredoxin the reaction mixture was aerated. At time intervals of 5 min 200 μl aliquots were mixed with 100 μl 2 M hydroxylamine (pH 7). After 10 min hydroxamic acids were determined according to [15].

2.2.3. Assay of acetyl coenzyme A formation

The reaction mixture contained: 3.0 M KCl, 50 mM Tris–HCl, pH 8.0, 1 mM Na_4EDTA, 40 μM *H. halobium* ferredoxin, 1 mM sodium pyruvate, 1 mM coenzyme A and enzyme extract (2–5 mg protein/ml). The reaction was started by addition of coenzyme A to the aerated sample. At intervals of 5 min 200 μl aliquots were taken. Acetyl coenzyme A was determined according to [16].

All chemicals used are commercially available. If not otherwise indicated, they were of analytical grade.

3. Results

3.1. *Cytochrome c reduction assay*

Reduced ferredoxin spontaneously transfers electrons to cytochrome *c*. This reaction has been used as an assay of NADPH-ferredoxin oxidoreductases [14,17,18] and can also be used to demonstrate the presence of α-ketoacid-ferredoxin oxidoreductases in *H. halobium*. Table 1 shows that cytochrome *c* reduction is dependent on coenzyme A, α-ketoacid, ferredoxin and the bacterial extract. Pyruvate, α-ketoglutarate and α-ketobutyrate are substrates of the reaction. The omission of ferredoxin from the incubation does not completely abolish the reaction. This is partly due to endogenous ferredoxin in the bacterial extract because the residual rate is reduced by one third when the enzymes are purified by gel filtration. The still remaining activity is presumably due to reduction of cytochrome *c* by the reduced enzyme itself or by superoxide arising from autoxidation of the reduced enzyme.

The reaction rate is proportional to the amount of enzyme present but is independent of cytochrome *c* concentration. Table 2 summarizes the Michaelis constants of ferredoxin and α-ketoacids in the three reactions. Because negligible amounts of reduced ferredoxin exist in the presence of oxygen (fig.2), the K_m values determined are those of oxidized ferredoxin. The enzymes can be fourfold purified by gel filtration on Ultrogel AcA 22 (LKB). Such preparations show a linear time dependence in the cytochrome *c* reduction assay. The bacterial extracts can be stored in the refrigerator for some time (table 1). Storage at −20°C enhances loss of activity. This is a common feature of many halophilic enzymes [19].

Table 1
Ferredoxin-dependent reduction of cytochrome *c* by various α-ketoacids

Substrate	Pyruvate	α-Keto-glutarate	α-Keto-butyrate
Complete	210	260	295
Complete, bacterial extract stored for 7 days at 5°C	183	212	258
Ferredoxin omitted	36	35	49
α-Ketoacid omitted	4	4	4
No addition of CoA	0	0	0
Bacterial extract omitted	0	0	0

The assays were performed as described in section 2.2.; the samples of the dialysed bacterial extract always contained 1.0 mg protein. Rates are given in pmol/s cytochrome *c* reduced

Table 2
Michaelis constants of the reactions (cytochrome *c* reduction assay)

Substrate	K_m (μM) Ferredoxin	α-Ketoacid
Pyruvate	30	100
α-Ketoglutarate	50	800
α-Ketobutyrate	40	70

The values were obtained from double reciprocal plots of the reaction rates and substrate concentrations. An enzyme preparation obtained by gel filtration (ferredoxin-free) was used. For K_m determination of ferredoxin the difference in rates with and without ferredoxin was plotted, and the α-ketoacids were present in saturating amounts. In K_m-determination of the α-ketoacids ferredoxin was present in 20 μM concentration

3.2. *Hydroxamic acid formation*

The activated acyl compounds formed in the three reactions can be estimated after conversion to hydroxamic acids. Opposite to the blue–green algal α-keto-acid-ferredoxin oxidoreductases [11,12], which are active in the presence of hydroxylamine, the halobacterial enzymes are rapidly inactivated by this agent at concentrations around 0.2 M. Hydroxylamine therefore can be used to stop the reaction efficiently.

Table 2 shows that the same substrate requirements are found for acyl derivative formation as for the cytochrome *c* reduction assay. The rates of the oxidation of pyruvate or α-ketobutyrate are identical in both assays within the limits of error. In the case of α-ketoglutarate only 60% of the cytochrome *c* reduction activity was found. This is probably due to a partial decomposition of succinyl coenzyme A during the incubation period.

3.3. *Identification of acetyl coenzyme A as the product of the pyruvate-ferredoxin oxidoreductase reaction*

Figure 1 demonstrates the formation of acetyl coenzyme A from pyruvate. Acetyl CoA was determined by the citrate synthase reaction [16]. Again the initial rates found by this assay and by the cytochrome *c* reduction assay are identical and the ferredoxin dependence of the reaction is evident.

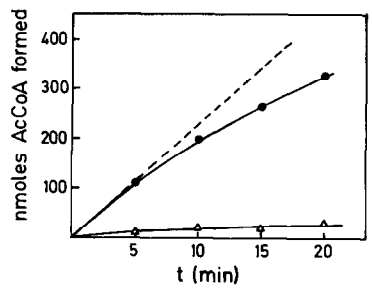

Fig.1. Formation of acetyl coenzyme A. The experiment was performed as described in 2.2 using 0.4 ml (0.7 mg protein) of the active fractions after gel filtration in the presence (•) and absence (△) of ferredoxin. The dotted line gives the reaction rate determined by the cytochrome *c* assay.

3.4. *Direct proof of ferredoxin reduction*

If a reaction mixture containing ferredoxin, α-ketoacid, coenzyme A and enzyme is placed in a cuvette of 1 mm light-path, the ferredoxin remains in the oxidised state until all oxygen in the solution is reduced, then the optical absorption decreases until complete reduction is achieved (fig.2). On complete reduction of *H. halobium* ferredoxin its absorption at 467 nm decreases from 8.9–4.1 $mM^{-1} cm^{-1}$ [2]. Omission of ketoacid, coenzyme A or enzyme as well as addition of 0.2 M hydroxylamine prevent the reduction of the ferredoxin.

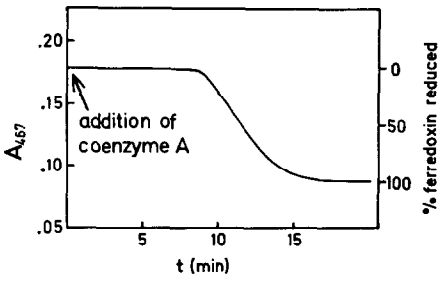

Fig.2. Reduction of *H. halobium* ferredoxin with α-ketoglutarate. The absorbance at 467 nm of 200 μl of the reaction mixture containing 50 mM Tris–HCl, pH 8.0, 1 mM Na_4 EDTA 200 μM ferredoxin, 5 mM α-ketoacid, 1 mM coenzyme A and 80 μl (0.14 mg protein) of the active fractions after gel filtration was recorded after starting the reaction by addition of the coenzyme A. A cuvette with 1 mm light-path was used.

Table 3
Formation of activated carboxylic acids as analyzed by the hydroxamic acid assay

Substrate	Pyruvate	α-Keto-glutarate	α-Keto-butyrate
Complete	1.56	1.20	2.41
Activity measured by cytochrome *c* reduction	1.52	1.96	2.28
Ferredoxin omitted	0.42	0.31	0.43
α-Ketoacid omitted	0.08	0.08	0.08
No addition of CoA	0.01	0.01	0.01
Enzyme omitted	0.07	0.05	0.10

The assays were performed as described in section 2.2.; the samples of the dialyzed bacterial extract always contained 10 mg protein. Rates are given in nmol/s hydroxamic acid formed. The cytochrome *c* reduction assays (line 2) were performed with the same substrate concentrations but only 1/10 of enzyme and coenzyme compared with the hydroxamic acid assay. Since the rate depends linearily on enzyme concentration and coenzyme A saturates the reaction at the applied concentration, the measured rates were multiplied with a factor of 10

4. Discussion

This paper presents evidence that oxidative decarboxylation of pyruvate, α-ketoglutarate and α-ketobutyrate is a ferredoxin-dependent process in *H. halobium* following the equation:

$$\alpha\text{-ketoacid} + CoA + 2\ Fd_{ox} \rightarrow Acyl\text{-}CoA + CO_2 + 2\ Fd_{red}$$

We could not detect pyridine nucleotide-dependent α-ketoacid oxidation in *H. halobium* cell homogenates in accordance with the results of other investigators [7]. We therefore conclude that the citric acid cycle in Halobacteria is dependent on ferredoxin. This again stresses the analogy of halobacterial ferredoxin and blue–green algal ferredoxins. Beside the sequence homology [3] the function as coenzymes in α-keto-acid decarboxylation instead of pyridine nucleotides is a common property of halobacterial and some blue–green algal ferredoxins [11,12,20].

Pyruvate-ferredoxin oxidoreductase and α-keto-glutarate-ferredoxin oxidoreductase are key enzymes of the reductive carboxylic acid cycle, which is very likely to bring about photosynthetic carbon dioxide reduction in *Chlorobium* [21,22]. The question arises, if Halobacteria, which are capable of bacteriorhodopsin dependent photophosphorylation [5,6], can also assimilate carbon dioxide. The redox equivalents necessary for this process may be furnished by a reversed electron flow at the expense of light energy and a suitable electron donor. Other prerequisites of a reductive carboxylic acid cycle, the existence of a citrate lyase and the reversibility of α-ketoacid oxidation under physiological conditions have not been investigated in *H. halobium*.

Recently it has been reported that illuminated bacteriorhodopsin-containing *H. halobium* cells incorporated carbon dioxide into acid stable products [23]. Because propionate stimulated this reaction, the authors assumed that α-ketobutyrate was formed from propionate and CO_2. However, no electron donor was added and the reaction products have not been determined. We think that a nonreductive carboxylation of propionate yielding succinate at the expense of the photosynthetically formed ATP is more likely to take place under the conditions described in [23]. Exactly this result has been obtained in the case of *Chlorobium thiosulfatophilum* cells illuminated in the absence of an electron donor [24].

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