INACTIVATION OF THE PYRUVATE FORMATE LYASE OF *CLOSTRIDIUM BUTYRICUM**

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1. Introduction

Recent investigations of the pyruvate formate lyase reaction have centered on the system in *Escherichia coli* [1–3], in *Streptococcus faecalis* [4] and in *Clostridium butyricum* [5]. In *E. coli* and *S. faecalis* the enzymes serve mainly to mediate acetyl-CoA generation for ATP synthesis in catabolism, while the clostridial lyase functions mainly to furnish formate for C_1-unit formation in anabolism.

The pyruvate formate lyases of *E. coli* and *C. butyricum* were reported to exist in interconvertible active and inactive forms [1, 5]. The activation process requires S-adenosyl-L-methionine (SAM) and a reducing system with flavodoxin or ferredoxin (Fd) as the physiological reductants [6]. An activating enzyme has been found in *E. coli* [1]. It was concluded that the mechanism of activation is an S-adenosyl-L-methionine controlled reduction of the lyase [1, 5]. The inactivation process has so far not been demonstrated.

This communication is a report on the inactivation of the pyruvate formate lyase in *C. butyricum*. In cell-free extracts the enzyme activity is lost in a time- and temperature-dependent process in the presence of reduced ferredoxin, when S-adenosyl-L-methionine is removed or its action inhibited by S-adenosyl-L-homocysteine (SAH). The inactivation is reversible. Thus the lyase is inactivated under the reductive conditions required for activation. These results suggest that in the activation process of the lyase, reduction may not be the sole chemical event.

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2. Methods

All chemicals were reagent grade. Enzymes, coenzymes and substrates were purchased from Boehringer Mannheim GmbH (Mannheim, Germany). [^{14}C]Formate was obtained from Amersham Buchler GmbH (Braunschweig, Germany). *C. butyricum* (ATCC 19398) and *C. pasteurianum* (ATCC 6013) were grown on the synthetic glucose/NH_4^+ medium described by Lovenberg et al. [7].

Crude extracts of *C. butyricum* were prepared by incubation of 2.5 g of frozen cells in 7.5 ml of 25 mM Tris-acetate, pH 8.5 containing 25 mM mercaptoethanol with 5 mg lysozyme and 0.5 mg deoxyribonuclease at 37° under hydrogen for 30 min and centrifugation at 40,000 *g* for 20 min. The cell suspension during the incubation and again the supernatant fluid after the centrifugation were adjusted to pH 7.3 with 1 N Tris base.

Extracts were made free of nucleotides including S-adenosyl-L-methionine by passing crude extract (10 ml) through a small column (Whatman, 1 cm diameter) filled with an anaerobic mixture of 2 g Dowex-2-acetate 200–400 mesh, and 100 mg charcoal previously treated with HCl and EDTA. Ferredoxin- and nucleotide-free extracts were obtained similarly using a DEAE-cellulose/Dowex-2-acetate/charcoal

column (1 cm diameter, 1 cm DEAE-cellulose layered on top of the 3 cm Dowex/charcoal mixture). Ferredoxin was prepared from *C. pasteurianum* by the method of Mortenson [8].

The activity of the pyruvate formate lyase was tested by the [^{14}C]formate–pyruvate exchange assay. The tests were carried out in Thunberg tubes, which had been repeatedly evacuated and filled with the desired gas. The main compartment contained 5 μmoles sodium pyruvate, 10 μmoles magnesium acetate, 25 μmoles mercaptoethanol, 0.5 μmoles S-adenosyl-L-homocysteine, 100 μmoles Tris-acetate, pH 7.5 and 4 mg extract protein; the side arm was filled with 20 μmoles sodium [^{14}C]formate (22,000 dpm/μmole); the total volume was 1 ml. From the extract incubations, in which the lyase was being activated or inactivated, aliquots of 4 mg protein were taken at the desired times and added against a stream of hydrogen to the assay mixture in the main compartment kept at 0°. At the end of the total activation–inactivation period, hydrogen was replaced with carbon monoxide. The test was then carried out simultaneously for all probes taken at different times; the assay was started by the addition of [^{14}C]formate from the side arm and was run for 10 min at 37° in a waterbath-shaker. The reaction was stopped with 1 ml 5% TCA and the precipitated protein was separated by centrifugation. After the addition of another 5 μmoles carrier-^{12}C-pyruvate the supernatant fluid containing the [^{14}C]pyruvate during the exchange was treated with 0.5 ml of a 0.15 M solution of 2,4-dinitrophenylhydrazine in 50% H_2SO_4. The precipitated pyruvate-2,4-dinitrophenylhydrazone was collected on a filter-paper-disk (Whatman nr. 42), washed with 10 ml water and counted on the disk in 20 ml Bray solution in a Packard 3380 liquid scintillation spectrometer.

The procedures used for activating or inactivating the lyase are described in the legends to the figures. The endogenous hydrogenase of the extracts was used to regenerate Fd_{red} under hydrogen (extrem rein; $0_2 < 1$ vpm) and Fd_{ox} under argon (extrem rein; 0_2, 1 vpm) or vacuum.

3. Results

When nucleotide-free extracts containing active pyruvate formate lyase were incubated at 37° under hydrogen with Fd_{red}, the enzyme activity was lost in a time-dependent process. It was completely restored by the addition of S-adenosyl-L-methionine. When then Fd_{red} was converted to Fd_{ox} by replacing the hydrogen atmosphere with a vacuum, the lyase was again inactivated. On reducing Fd_{ox} back to Fd_{red} by the reintroduction of a hydrogen atmosphere, the enzyme was reactivated to normal levels (fig. 1). The lyase activity was stable only when both S-adenosyl-L-methionine and Fd_{red} were present. Thus inactivation was observed under the reductive conditions required for activation as well as under oxidative conditions. Both types of inactivation were reversible. The finding that the rates of the "reductive" and the "oxidative" inactivation were essentially equal, indicates that a common non-oxidative step may be operative in both mechanisms.

The activation process by S-adenosyl-L-methionine and Fd_{red} was reversed to an inactivation process by the addition of a solution of S-adenosyl-L-homocysteine, while it was seen to continue normally when buffer was

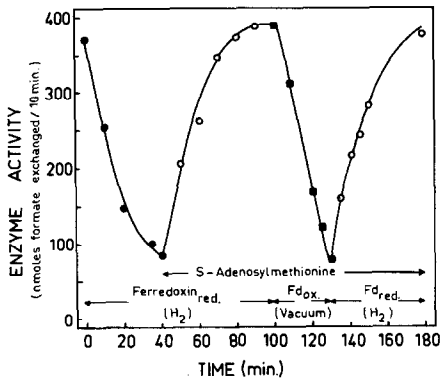

Fig. 1. Reversible inactivation of the pyruvate formate lyase of *C. butyricum* under reductive and oxidative conditions. The lyase was first activated by incubating crude extract (10 ml) with 0.5 mM S-adenosyl-L-methionine (SAM) and 0.3 mg protein/ml ferredoxin (Fd) at 37° for 40 min under hydrogen (= Fd_{red}). The thus activated crude extract was rapidly cooled to 0° and then passed over a Dowex-2-acetate/charcoal column to remove all nucleotides and incubated at 37° under hydrogen (= Fd_{red}). At 40 min 0.5 mM S-adenosyl-L-methionine was added against a stream of hydrogen. The hydrogen atmosphere was replaced with a vacuum (= Fd_{ox}) at 100 min and reintroduced at 130 min. At the times indicated 200 μl samples were withdrawn, added to the test mixture kept at 0° and assayed as described under Methods.

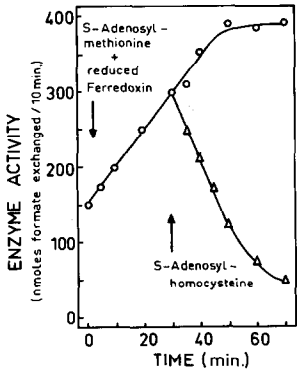

Fig. 2. Reversal of the S-adenosylmethionine- and reduced ferredoxin-dependent activation of the pyruvate formate lyase of *C. butyricum* to inactivation by S-adenosylhomocysteine. The crude extract (10 ml) was first passed over a DEAE-cellulose/Dowex-2-acetate/charcoal column to remove ferredoxin and all nucleotides and then incubated at 37° with 0.5 mM S-adenosyl-L-methionine and 0.3 mg protein/ml ferredoxin under hydrogen (= Fd_{red}). At 30 min one half of the extract incubation (5 ml) was anaerobically mixed with 0.5 ml 5 mM S-adenosylhomocysteine, the other half with 0.5 ml 50 mM Tris-acetate buffer, pH 7.3. Samples were taken and tested as described in fig. 1.

added in amounts equivalent to the S-adenosyl-L-homocysteine solution used (fig. 2). Apparently the activation observed was a net effect resulting from the simultaneous activation and inactivation of the enzyme. This continuous turnover reached a plateau when the velocity of activation and inactivation became equal. The predominating activation could be interrupted with S-adenosyl-L-homocysteine, a S-adenosyl-L-methionine antagonist [5], and be converted to a predominating inactivation. The S-adenosyl-L-homocysteine induced conversion of activation to inactivation was observed also in the presence of ferrous/dithiol complexes used as oxygen scavenging agents. Evidently oxygen was not involved in this process. Again it can be concluded that a non-oxidative step may be functioning in the inactivation of the lyase.

Activation and inactivation were carried out routinely at 37°: they could not be demonstrated at 0°. The time- and temperature-dependence may be taken as preliminary evidence that the interconversion of the clostridial lyase is a chemical rather than a physical process and that, similar to the activation in *E. coli*, it is protein catalyzed. Direct proof cannot be obtained as long as the substrate of the interconversion, the pyruvate formate lyase, is not available in a purified form due to its instability even under reductive conditions.

4. Discussion

It was proposed that the activation of the pyruvate formate lyase occurs by a reduction of the enzyme and that this process is under allosteric control by S-adenosyl-L-methionine [1, 5]. The biological usefulness of such an effect by S-adenosyl-L-methionine, however, is not understood. In *C. butyricum*, having an anabolic lyase involved in C_1-unit generation, the supposed positive control by S-adenosyl-L-methionine is functionally contradictory, because a high level of S-adenosyl-L-methionine should indicate a replenished C_1-pool and thus be a signal for inactivation rather than activation. In *E. coli*, having a catabolic lyase involved in ATP generation, the supposed allosteric effect of S-adenosyl-L-methionine is functionally incomprehensible, since no regulatory relation between ATP generation and C_1-anabolism is apparent.

In this communication evidence has been presented showing that an oxidative and a non-oxidative chemical process may participate in the inactivation of the pyruvate formate lyase of *C. butyricum*. Consequently the ferredoxin dependent reduction of the enzyme is probably not the sole chemical event in the activation; a non-reductive step may be also involved.

A model, in which such reactions participate, can be visualized: The lyase is first reduced and then activated by alkylation with S-adenosyl-L-methionine; the active enzyme is inactivated by dealkylation and then oxidized. Methylation, adenosylation or aminocarboxypropylation should be considered as possible modifying actions of S-adenosyl-L-methionine. Methylation of the *E. coli* lyase could not be demonstrated; the other modes of alkylation do not appear to have been tested as yet [1]. Alkylation and dealkylation would be the missing non-reductive and non-oxidative processes. In this model S-adenosyl-L-methionine would function as a modifying substrate rather than as an allosteric effector.

The now proposed alkylating action of S-adenosyl-L-methionine would provide a unifying concept applicable to both *C. butyricum* and *E. coli* in that alkylation may be a means of stabilizing reduced functional

groups. The different metabolic function of the enzyme in the two organisms would suggest that the alkylation–dealkylation be controlled by different effectors on which nothing is known at present.

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References

[1] J. Knappe, J. Schacht, W. Möckel, Th. Höpner, H. Vetter and R. Edenharder, European J. Biochem. 11 (1969) 316.
[2] J.R. Chase and J.C. Rabinowitz, J. Bacteriol. 96 (1868) 1065.
[3] H. Nakayama, G.G. Miswinter and L.O. Krampitz, Arch. Biochem. Biophys. 143 (1971) 526.
[4] D.G. Lindmark, P. Paolella and N.P. Wood, J. Biol. Chem. 244 (1969) 3605.
[5] R.K. Thauer, F.H. Kirchniawy and K.A. Jungermann, European J. Biochem. 27 (1972) 282.
[6] H. Vetter and J. Knappe, Z. Physiol. Chem. 352 (1971) 433.
[7] W. Lovenberg, B.B. Buchanan and J.C. Rabinowitz, J. Biol. Chem. 238 (1963) 3899.
[8] L.E. Mortenson, Biochim. Biophys. Acta 81 (1964) 71.