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Pyruvate-formate-lyase-deactivase and acetyl-CoA reductase activities of *Escherichia coli* reside on a polymeric protein particle encoded by *adhE*

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A 4.8 kb DNA-fragment was cloned and sequenced encompassing the structural gene of PFL-deactivase (2.7 kb) and 2 kb of the 5' flanking region that contains the elements for anaerobic induction. A mutant lacking deactivase was shown to require exogeneus electron acceptors for anaerobic growth with glucose. This revealed the identity of PFL-deactivase with the alcohol and acetaldehyde dehydrogenases of *E. coli*. The multienzyme represents a homopolymeric protein (~ 40 × 96 kDa) requiring Fe^{2+} for all functions.

Fe^{2+}-activation; Spirosome; Nucleotide sequence; Anaerobic induction

1. INTRODUCTION

Glucose fermentation by *E. coli* cells involves transformation of pyruvate into acetyl-CoA and formate as the key step that initiates the characteristic terminal portion of the glycolytic route in that organism. The catalyst, pyruvate formate-lyase (PFL), is distinguished by containing a stable organic free radical, probably localized at an aliphatic amino acid residue, which functions as a coenzyme for substrate conversion. The radical results from an AdoMet and dihydroflavodoxin-dependent posttranslational protein modification, catalyzed by PFL-activase. Another feature of pyruvate formate-lyase is its prominent anaerobic induction, which involves the transcription factor FNR (for a recent review, see [1]).

Previous studies (reported in [1,2]; to be published in detail elsewhere) demonstrated a second converter enzyme, termed PFL-deactivase, in *E. coli* cell extracts; this catalyzes quenching of the PFL radical by using Fe^{2+}, NAD and CoA as cofactors (Eqn. 1):

$$PFL_a(\text{radical form}) \xrightarrow{Fe^{2+},\ NAD,\ CoA} PFL_i(\text{nonradical form}) \quad (1)$$

The deactivase, which is anaerobically induced to the same level as PFL, was identified as having an intriguing homopolymer structure, where about 40 subunits of 100 kDa are helically assembled into a rod of about 130 nm in length.

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We here report the cloning and sequencing of the structural gene of PFL-deactivase and studies of a chromosomal null mutant, undertaken to elaborate the physiology of PFL backconversion to the nonradical form. This surprisingly uncovered the fact that the polymeric enzyme harbors the alcohol plus acetaldehyde/CoA dehydrogenase activities of *E. coli*.

2. MATERIALS AND METHODS

2.1. *Materials*

[^{35}S]dATPαS was obtained from Amersham Buchler. The DNA sequencing kit (Sequenase version 2.0) was from USB Corp.; pUC/M13 sequencing primers and vector pUC19 were from Boehringer Mannheim. Vector pBT306.1 was a gift of Dr. G. Schumacher. Restriction enzymes and other enzymes used for DNA recombinant DNA procedures were from New England Biolabs or Boehringer Mannheim. Other enzymes and biochemicals were from Boehringer Mannheim or Serva. Immobilon for western blotting and nitrocellulose HATF for replica plating were from Millipore.

2.2. *Bacterial strains and plasmid constructs*

Strains were *E. coli* K12 (ATCC10798), MC4100, JM109 and IL746 (MC4100 with Cm^r inserted into *adhE*); *Pseudomonas putida* mt-2 KT2440. Plasmids contained the 4.8 kb fragment harboring the deactivase gene (*adhE*) ligated into pUC19 (pIL343) or pBT306.1 (pSH343). Antibiotics were applied to growth media as follows: ampicillin, 100 mg/l; kanamycin, 50 mg/l; chloramphenicol, 20 mg/l.

2.3. *DNA methods*

Recombinant DNA methods were as compiled by Sambrook et al. [3]. Nucleotide sequencing, by the Sanger method, employed plasmid DNA from exonuclease III generated subclones of pIL343 and covered both directions. Insertion of the Cm^r gene (from pACYC184) for constructing IL746 was according to [4].

2.4. *Isolation and characterization of PFL-deactivase*

20 g of *E. coli* IL746/pSH343 cells, grown anaerobically at pH 7 on glucose (15 g/l) minimal medium to the stationary phase and stored at −60°C, were suspended in 40 ml 50 mM Mops/KOH, pH 8.5, con-

taining 5 mM EDTA, 5 mM dithioerythritol and 0.3 mM phenylmethanesulfonyl fluoride, and extracted, at 4°C, by sonication. After centrifugation (12 000 × g, 1 h), the extract (1400 mg protein) was adjusted to pH 7.3, and 1.4 ml 10% (w/v) Polymin G35 (BASF) was added; the precipitate was discarded. The supernatant (40 ml; 950 mg) was carried through gel chromatography on Ultrogel AcA 44 (LKB; 5 cm^3 × 80 cm) with 50 mM Mops/KOH, pH 7.65, plus 0.1 M KCl at a flow-rate of 4 cm/h. Deactivase was collected with the first 60 ml portion of the eluate that appeared after the void volume. This sample (175 mg) was gel-filtered into 45 mM Tris-HCl, pH 8.3, diluted to 175 ml and immediately pumped onto DEAE-cellulose (DE 52 Whatman; 5 cm^3 × 12 cm), followed by washing with 25 ml starting buffer (15 cm/h). A linear gradient (180 ml) to 300 mM KCl in the same buffer was applied (8 cm/h). Homogenous enzyme (usually 90–110 mg) was collected with the first protein peak which eluted at about 90 mM KCl. The sample was pressure concentrated, using an Amicon XM 50-membrane, and transferred into 20 mM Mops-KOH, pH 7.6 (containing 0.1 mM EDTA) for storage. The activity was stable at −60°C for up to one year. Application of this procedure to *E. coli* K12 wild-type cells yielded 10–15 mg deactivase.

Edman degradation found A-V-T-N-V-A- as the N-terminal sequence. Size measurements gave 100 kDa for the constituent polypeptide (SDS-PAGE) and a Stokes' radius of 110–150 Å for the native protein (AcA 22 gel chromatography). The p*I* was 7.0.

Electron microscopy, kindly conducted by Dr. W. Herth, employed a Philips CM10 instrument at an acceleration voltage of 100 kV. The deactivase sample (50 μg/ml) in anaerobic buffer, pH 7.5, containing 2 mM NAD and 0.3 mM $Fe(NH_4)_2(SO_4)_2$ was adsorbed to a carbon-coated grid under argon (15 s) and finally stained with 1% uranyl acetate, pH 4.5.

2.5. *Enzyme assays*

All assays used oxygen-free 0.1 M Mops-KOH, pH 7.5, with 6 mM dithiothreitol, 5 mM $MgSO_4$ and 0.3 mM $Fe(NH_4)_2(SO_4)_2$ as standard buffer and were performed under argon at 30°C. Alcohol dehydrogenase and acetyl-CoA reductase were measured spectroscopically with 0.4 mM NADH and 10 mM acetaldehyde or 0.2 mM acetyl-CoA in 1 ml final volume. Units (U) were defined as μmol NADH oxidized per min. For PFL-deactivase, the assay contained 2 mM NAD, 5 μM CoA, and 17 μg (100 pMol, 3.5 U) radical form of pyruvate formate-lyase (PFL_a) in 0.35 ml standard buffer. The reaction was followed via determining residual PFL activity [5]. 1 mU deactivase was defined as the amount that catalyzed quenching of 1 nMol PFL-radical per min. PFL_a was prepared by previous procedures [6].

Enzyme activities of crude cell extracts were determined on previously gel-filtered (Sephadex G-25) samples. All protein values are by the biuret method.

2.6. *Immuno techniques*

Screening of transformed JM109 cells employed replicas of agar colonies not larger than 1 mm (to hinder anaerobiosis and thus keep the background-signal from the chromosome-encoded PFL-deactivase at a low level). After cell lysis [7] overproducers were immunodetected with anti PFL-deactivase antiserum from rabbit, using protein A/horseradish peroxidase conjugate (BioRad) plus 4-chloro-1-naphthol.

3. RESULTS

3.1. *Molecular cloning and sequencing of a 4.8 kb fragment specifying PFL-deactivase (*adhE*)*

Chromosomal DNA-fragments of *E. coli* K12, generated by partial *Sau*3A digestion and gel electrophoretic size fractionation (4–6 kb), were used to construct a plasmid library by ligation into the pUC19 vector and transformation into *E. coli* JM109. Immuno-screening of about 8000 transformants with antiserum against PFL-deactivase obtained one positive clone. Its recombinant plasmid, pIL343, was analyzed by restriction enzyme mapping (Fig. 1) and nucleotide sequencing (4766 bp) of the total insert (Fig. 2).

We identified an open reading frame coding for a polypeptide of 891 amino acid residues (96 010 Da) that matched with N-terminal sequence and molecular weight data determined for the purified PFL-deactivase. No significant homology with nucleotide sequences deposited in the GenBank/EMBL data bank was found at that time. But after revealing the dehydrogenase functions of PFL-deactivase (section 3.3), we became aware of the previously published sequence of *adhE* [8], coding for *E. coli* alcohol dehydrogenase. It proved to be identical with our nucleotide sequence data for the deactivase gene along a stretch of 2759 bp.

Within the 2 kb region upstream of the structural gene (see Fig. 2), a single DNA sequence of strong similarity to the consensus promoter sequence is located around nt −690. This is preceded, at a distance of about 70 bp, by a DNA stretch (TTGCC-N_4-ATCAT) of significant similarity to the consensus sequence (TTGAT-N_4-ATCAA) for FNR protein recognition [9]. These structures lying far upstream could be responsible for the anaerobic regulation of deactivase (see section 3.2). Support for this suggestion stems from the reported lack of anaerobic induction of alcohol dehydrogenase synthesis from plasmid pHIL8 [8] wherein the upstream segment of *adhE* comprises as much as 400 bp.

3.2. *Expression of the plasmid-borne protein*

By insertion of the Cm^r gene and homologous recombination via P1 [4], an *E. coli* mutant lacking

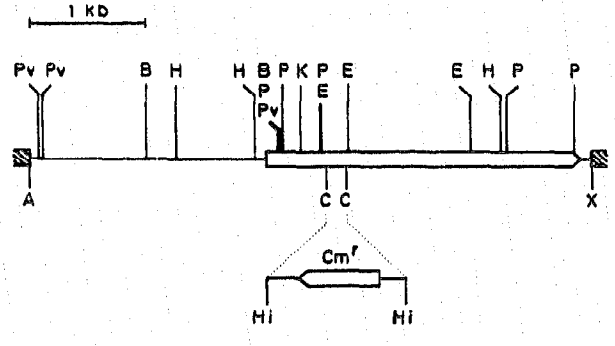

Fig. 1. Restriction map of plasmid pIL343 insert. Major endonuclease restriction sites and the location of the deactivase structural gene (*adhE*) are shown. Hatched boxes indicate connections from vector pUC19. The data were deduced from the nucleotide sequence and experimentally verified for A, Ba, B, C, Ec, H, P and X. Alignment (at 27.4 min) with the physical map of the *E. coli* chromosome [15] indicates anticlockwise gene polarity. The lower panel denotes the mode of Cm^r insertion for constructing the mutant strain IL746. Abbreviations: A, *Ava*I; Ba, *Bam*HI; B, *Bgl*I; C, *Cla*I; Ec, *Eco*RI; E, *Eco*RV; H, *Hin*dIII; Hi, *Hin*P1; K, *Kpn*I; P, *Pst*I; Pv, *Pvu*II; X, *Xba*I.

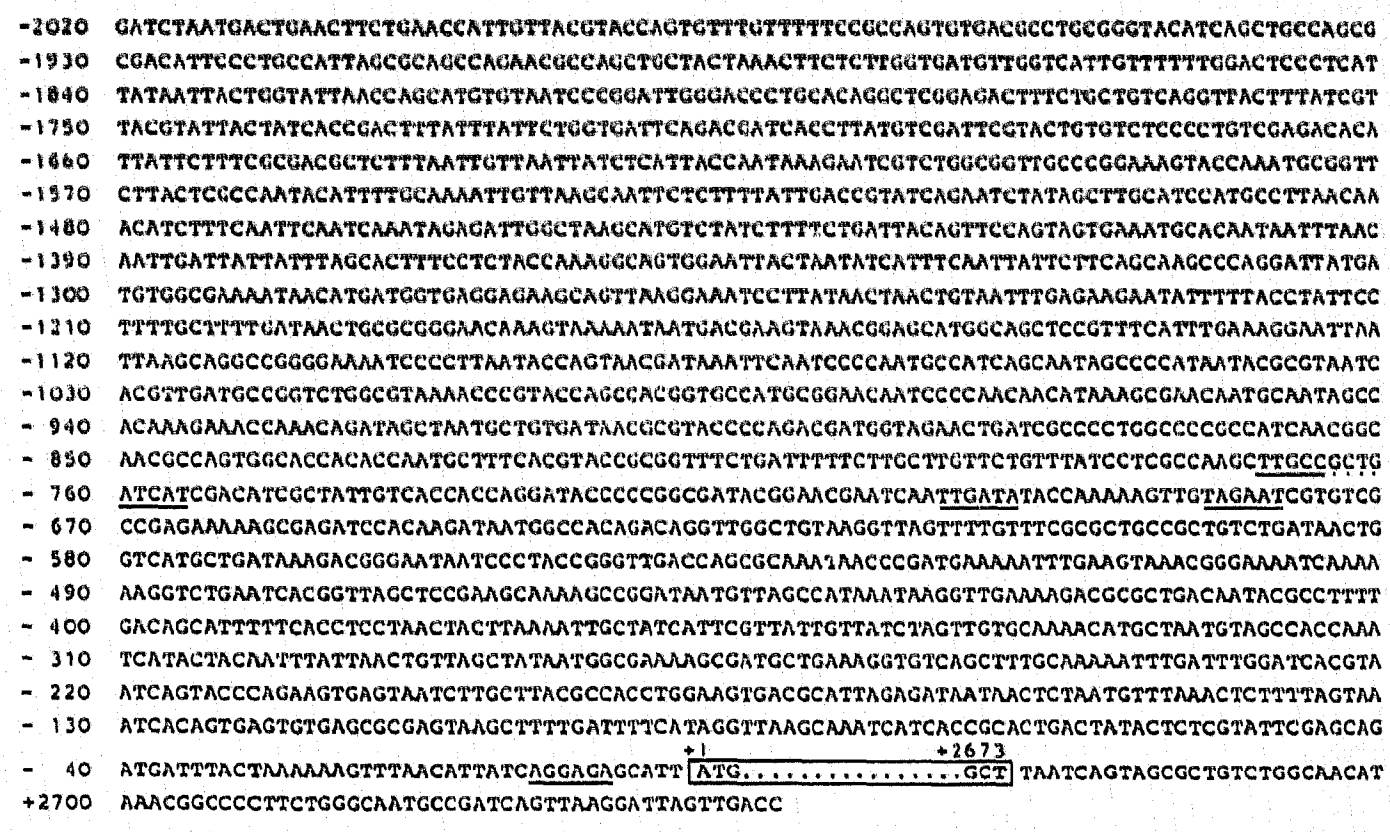

Fig. 2. Nucleotide sequence of pIl343 insert encompassing the deactivase gene (*adhE*). Numbering of nucleotides is relative to the adenine residue of the initiator methionine of the coding region of deactivase (boxed stretch). The structural gene is idental to *adhE*, the nucleotide sequence of which (from −83 to +2676) is published in [8]. Underlined stretches refer to a potential FNR-binding-site (−769 to −756), the −35 and −10 promoter consensus regions (beginning with −702 and −683) and the Shine Dalgarno sequence (−11). The depicted 2 kb-stretch also shows three parallel open reading frames (−1817 to −1449; −1578 to −1285; −889 to −590) and one antiparallel ORF (−576 to −1121); they lack a typical Shine Dalgarno sequence. Beginning with nt −1816, the complementary strand has a further ORF, which represents part of *oppA* (see [16]).

PFL-deactivase was constructed. This strain (IL746) was used primarily as a host for verifying the expression of the cloned structural gene. Plasmid pSH343, comprising the 4.8 kb insert of pIL343 religated into the shuttle vector pBT306.1, was applied to these experiments. Substantial amounts of deactivase were synthesized in IL746/pSH343 cells grown in glucose minimal medium. The level was increased about 10-fold at anoxic conditions (Fig. 3), which suggests that the cloned DNA fragment harbored all sequence elements essential for anaerobic regulation. (For wild-type *E. coli* K12 cells, immunotitrations of the chromosome-directed PFL-deactivase found an anaerobic induction/derepression factor of 8–12). It is interesting to note that the protein was produced also in pSH343 transformed cells of the obligately aerobic *Ps. putida* (data not shown).

To assess the identity of the gene product we purified the protein from batch-cultured IL746/pSH343 cells. The catalytic properties as well as the striking ultrastructure, elaborated by gel chromatography and electron microscopy (Fig. 4), were indistinguishable from that of the chromosome-specified protein.

3.3. *Anaerobic growth phenotype of the null mutant (IL746)*

To investigate physiological consequences of a defective PFL-deactivase, bacterial growth in glucose minimal medium was studied in chemostat cultures maintained at pH 7. IL746 cells grew normally (1.2 h doubling time) with oxygen applied, but proved unable to grow anaerobically unless an electron acceptor substrate was supplemented. With 0.1 M nitrate, the doubling time (1.5 h) was like that of the parental strain MC4100 at normal or nitrate supplemented anoxic conditions.

The observed phenotype was that of the *ana* mutant of *E. coli* [10] which was previously characterized as lacking the enzyme activities for endogenous reoxidation of the glycolytic NADH by reducing acetyl-CoA to ethanol:

$$\text{NADH} + \text{acetyl-CoA} \rightleftharpoons \text{NAD} + \text{acetaldehyde} + \text{CoA} \quad (2)$$

$$\text{NADH} + \text{acetaldehyde} \rightleftharpoons \text{NAD} + \text{ethanol} \quad (3)$$

We found then that the IL746 cells also lacked these activities (Table I), thus indicating their association with the PFL-deactivase protein.

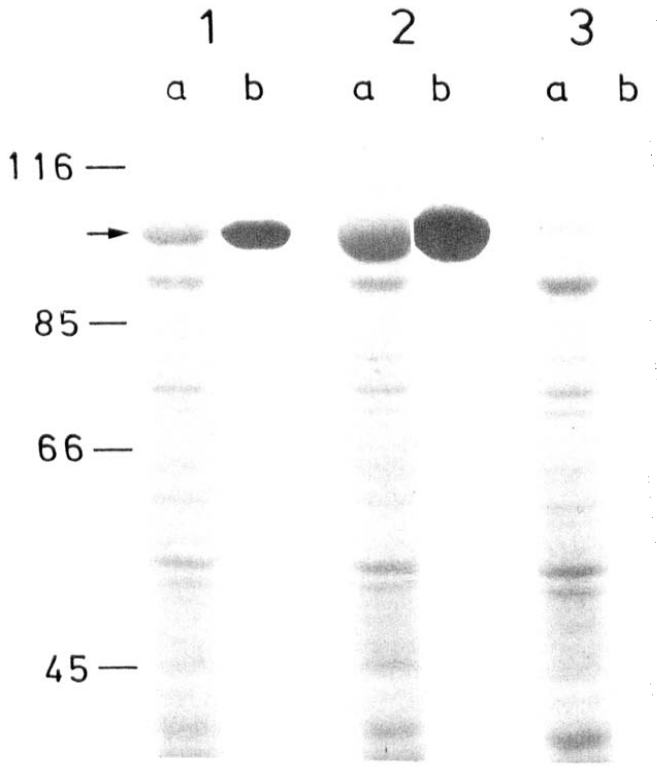

Fig. 3. Anaerobically induced enzyme production from the cloned 4.8 kb fragment. About 5×10^7 cells of strain IL746/pSH343, grown either aerobically (Expt. 1) or anaerobically (Expt. 2) on glucose (pH 7, 37°C), were lysed with 2% SDS (4 min, 100°C). The solubilized material was subjected to SDS-PAGE (8%), electroblotted and Coomassie-stained (lanes a) or immuno-stained (lanes b). Expt. 3 employed untransformed IL746 cells, grown anaerobically with 0.1 M nitrate. (Figures (kDa) indicate marker protein migration.)

3.4. *Multiple Fe^{2+} dependent redox functions of the polymeric enzyme*

Assays on the isolated enzyme confirmed this suggestion. The activity data, summarized in Table I, established unequivocally that the catalytic capacity of acetyl-CoA to ethanol reduction of *E. coli* cells is due to the same protein that we purified through its property of quenching the PFL radical.

Since the deactivase function is strictly Fe^{2+} dependent, we examined whether metals could also be involved in the dehydrogenase functions. Both activities, with acetyl-CoA or acetaldehyde as substrates, proved to be significantly and specifically stimulated by Fe^{2+} (Table II). Half-maximum activity was achieved with about one equivalent of free Fe^{2+} applied per enzyme promoter in the assay. When the assays were performed in the reverse directions, the metal effect was only observed with the ethanol substrate, not with acetaldehyde/CoA.

Consistent with the thermodynamic data for reactions (2) and (3), the spectroscopic assays determined virtually complete conversion of acetyl-CoA to ethanol from a small excess of NADH applied (Fig. 5). The

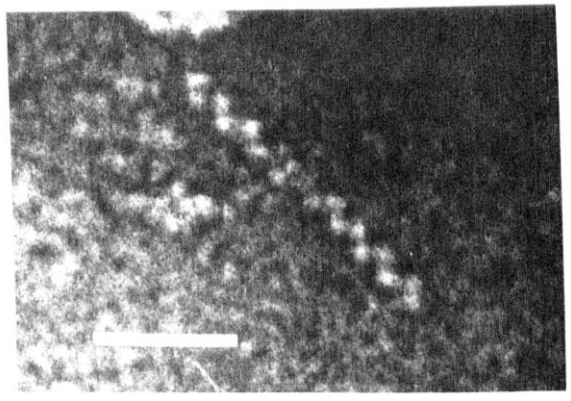

Fig. 4. Electron micrograph of the negatively stained PFL-deactivase/acetyl-CoA reductase protein. The bar indicates 50 nm. The depicted specimen is representative of the large majority of molecules observed (size distribution 60–220 nm in length).

overall process of thioester reduction to the alcohol stage, catalyzed by a single protein, resembles the reaction of HMG-CoA reductase, but at variance with the latter enzyme, the 'acetyl-CoA reductase' can readily accept the intermediary aldehyde as substrate.

For the deactivase function, NAD in its oxidized form is required, and the PFL radical quenching process is unidirectional. We found now that the reaction

Table I

Catalytic activities

	PFL-deactivase (mU/mg)	Acetyl-CoA reductase (U/mg)	Ethanol dehydrogenase (U/mg)
Chromosome encoded enzyme	11.0	9.6	9.9
Plasmid encoded enzyme	11.0	10.4	9.9
E. coli K12 cell extract	0.36	0.2	0.23
E. coli IL746 cell extract	≤0.006	≤0.004	≤0.006

Assays were performed in the presence of 0.3 mM Fe^{2+} at pH 7.5 and 30°C.

Table II

Activation by Fe^{2+}.

Additions	Catalytic activity: Acetyl-CoA reductase	PFL-deactivase
0.1 mM EDTA	9	–
None	17	≤5
0.03 μM Fe^{2+}	56	–
0.03 mM Fe^{2+}	*100*	*18*
0.3 mM Fe^{2+}	100	100
0.03 mM Zn^{2+}	15	–
0.03 mM Co^{2+}	18	≤5
0.03 mM Mn^{2+}	17	–

Measurements were by the standard assays (Fe^{2+} omitted) with variations as indicated, using enzyme concentrations of 4 μg/ml (acetyl-CoA reductase) and 3 μg/ml (deactivase). Activity values are relative to that at standard conditions with 0.3 mM Fe^{2+} = 100. Data (not shown) for acetaldehyde reduction were similar to that with acetyl-CoA.

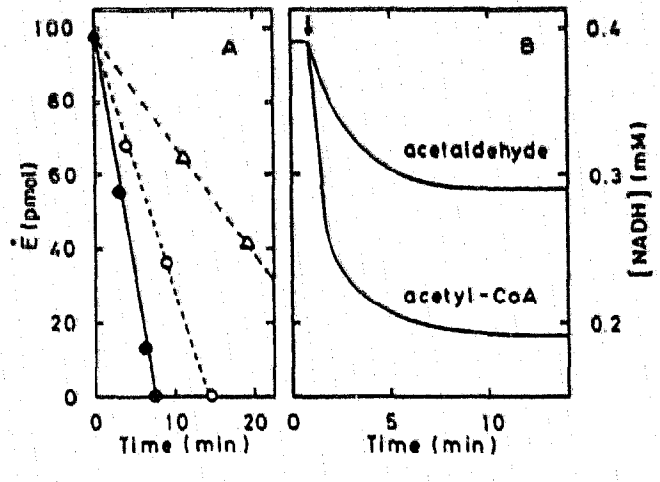

Fig. 5. Conversion of PFL from the radical to the nonradical form (A) and reduction of acetyl-CoA/acetaldehyde (B). For reaction conditions, see Materials and Methods. Expt. A employed 98 pmol PFL_a ($\dot{E}$), 1.8 μg purified enzyme, 1.1 mM NAD and NADH as follows: 0 mM (•), 0.17 mM (○) or 0.86 mM (□). Expt. B employed 0.1 μmol acetyl-CoA or acetaldehyde and 19 μg purified enzyme; NADH consumption was registrated photometrically.

rate is drastically lowered when small fractions of NADH are simultaneously present (Fig. 5A). This should have a direct bearing on the physiological usage of the various catalytic functions of the multienzyme.

4. DISCUSSION

Association of the two dehydrogenase activities (eqns. (2) and (3) of *E. coli* with a single, large-sized protein has long been suggested (for a review, see [11]) and was more recently ascribed to the 96 kDa polypeptide encoded by *adhE* [8]. We now have delineated a rod-shaped polymer structure comprising about 40 identical subunits and found that its redox functions are specifically activated by Fe^{2+}. The protein is most probably identical with the 'spirosomes' detected in many obligate and facultative anaerobes (see [12]).

In glucose fermentation, NAD regeneration by the polymeric enzyme immediately follows pyruvate conversion to acetyl-CoA. Its indispensability in the absence of exogenous electron acceptors is a direct consequence of the PFL reaction. Both proteins, the polymeric enzyme and pyruvate formate-lyase, are prominent anaerobic gene products with induction factors of 10–15. They represent spots 'H 97.3' and 'G 74', respectively, of the 2D map of anaerobic polypeptides [13]. The nucleotide sequence motifs bound in the 5′ upstream region of *adhE* could suggest a transcriptional control by the FNR protein [9] as previously delineated for the *pfl* gene [14].

In anaerobically growing bacteria at pH 7, PFL exists predominantly in the active radical form which becomes converted back to the nonradical form upon exhaustion of the glucose supply [5]. We expect that other physiological conditions should occur where the polymeric enzyme will exert its PFL deactivating function. This could possibly be switched on by an increased intracellular NAD/NADH ratio whereupon metabolic fluxes would be rerouted (e.g. glycolysis towards lactate). The chemical mechanism of quenching the protein radical of pyruvate formate-lyase is another intriguing property of the polymeric enzyme to be investigated.

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REFERENCES

[1] Knappe, J. and Sawers, G. (1990) FEMS Microbiol. Rev. 75, 383–398.
[2] Kessler, D. and Knappe, J. (1988) 14th Int. Congr. Biochem. (Prague), Abstract TH 367.
[3] Sambrook, J., Fritsch, E.F. and Maniatis, T. (1989) Molecular Cloning: A Laboratory Manual, Cold Spring Harbor Laboratory, Cold Spring Harbor, NY.
[4] Winans, S.C., Elledge, S.J., Heilig Krueger, J. and Walker, G.C. (1985) J. Bacteriol. 161, 1219–1221.
[5] Conradt, H., Hohmann-Berger, M., Hohmann, H.P., Blaschkowski, H.P. and Knappe, J. (1984) Arch. Biochem. Biophys. 228, 133–142.
[6] Unkrig, V., Neugebauer, F.A. and Knappe, J. (1989) Eur. J. Biochem. 184, 723–728.
[7] Helfman, D.M., Feramisco, J.R., Fiddes, J.C., Thomas, G.P. and Hughes, S.H. (1988) Proc. Natl. Acad. Sci. USA 80, 31–35.
[8] Goodlove, P.E., Cunningham, P.R., Parker, J. and Clark, D.P. (1989) Gene 85, 209–214.
[9] Spiro, S. and Guest, J.R. (1990) FEMS Microbiol. Rev. 75, 399–428.
[10] Pascal, M.C., Chippaux, M., Abou-Jaoudé, A., Blaschkowski, H.P. and Knappe, J. (1981) J. Gen. Microbiol. 124, 35–42.
[11] Clark, D.P. (1989) FEMS Microbiol. Rev. 63, 223–234.
[12] Matayoshi, S., Oda, H. and Sarwar, G. (1989) J. Gen. Microbiol. 135, 525–529.
[13] Smith, M.W. and Neidhardt, F.C. (1983) J. Bacteriol. 154, 336–343.
[14] Sawers, G. and Böck, A. (1989) J. Bacteriol. 171, 2485–2498.
[15] Kohara, Y., Akiyama, K. and Isono, K. (1987) Cell 50, 495–508.
[16] Kashiwagi, K., Yamaguchi, Y., Sakai, Y., Kobayashi, H. and Igarashi, K. (1990) J. Biol. Chem. 265, 8387–8391.