(*Washington, D.C.*) *200*, 394–402.
Schmid, C. W., & Jelinek, W. R. (1981) *Science* (*Washington, D.C.*) (in press).
Schmookler-Reis, R. J., & Goldstein, S. (1980) *Cell* (*Cambridge, Mass.*) *21*, 739–746.
Shapiro, J. A. (1979) *Proc. Natl. Acad. Sci. U.S.A. 76*, 1933–1937.
Smith, C. A., & Vinograd, J. (1972) *J. Mol. Biol. 69*, 163–178.
Southern, E. M. (1975) *J. Mol. Biol. 98*, 503–517.
Stanfield, S., & Helinski, D. R. (1976) *Cell* (*Cambridge, Mass.*) *9*, 333–345.
Stanfield, S. W., & Lengyel, J. A. (1979) *Proc. Natl. Acad. Sci. U.S.A. 76*, 6142–6146.
Thayer, R. E., Singer, M. F., & McCutchan, T. F. (1981) *Nucleic Acids Res. 9*, 169–181.
Wakamuya, T., McCutchan, T., Rosenberg, M., & Singer, M. (1979) *J. Biol. Chem. 254*, 3584–3591.
Wu, J. C., & Manuelidis, L. (1980) *J. Mol. Biol. 142*, 363–386.
Young, M. W. (1979) *Proc. Natl. Acad. Sci. U.S.A. 76*, 6274–6278.

In Vitro Synthesis of the Membrane-Bound D-Lactate Dehydrogenase of *Escherichia coli*†

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ABSTRACT: Synthesis of the membrane-bound, flavin-linked D-lactate dehydrogenase of *Escherichia coli* has been studied by using a recombinant plasmid containing the *dld* gene [Young, I. G., Jaworowski, A., & Poulis, M. (1982) *Biochemistry* (following paper in this issue)]. Expression of the cloned *dld* gene was achieved either in vivo with transformed minicells or in vitro with a fractionated transcription/translation system. In both instances, a product is observed that is specifically immunoprecipitated by γ-globulin prepared against the purified enzyme and comigrates with authentic D-lactate dehydrogenase on sodium dodecyl sulfate–polyacrylamide gel electrophoresis. Furthermore, the product is catalytically active and binds to membrane vesicles during or after synthesis. Thus, it seems likely that the protein is synthesized in mature form and binds to the membrane without a leader peptide sequence. Interestingly, addition of flavin adenine dinucleotide to the in vitro reaction mixtures causes a 2-fold increase in the synthesis of the enzyme, suggesting that the cofactor plays a regulatory role in the synthesis of the apoprotein. Finally, L factor, a protein involved in regulation of protein elongation, has an inhibitory effect on the expression of the *dld* gene and a stimulatory effect on the expression of the *ndh* gene (encoding NADH dehydrogenase).

Substrate oxidation via a membrane-bound respiratory chain drives the active transport of a wide variety of solutes in right-side-out membrane vesicles prepared from many bacteria (Kaback, 1972, 1974; Owen & Kaback, 1978, 1979a,b) by mechanisms in which an electrochemical gradient of protons ($\Delta\bar{\mu}_{H^+}$,[1] interior negative and alkaline) is the immediate driving force (Mitchell, 1963, 1968, 1973; Kaback, 1976; Harold, 1977; Konings & Boonstra, 1977). Furthermore, for reasons that are not fully understood, although the vesicles have the capacity to oxidize a number of substrates, generation of $\Delta\bar{\mu}_{H^+}$ is relatively substrate specific (Kaback, 1972, 1974). Thus, in vesicles from *Escherichia coli* and *Salmonella typhimurium*, D-lactate serves as the most effective physiological electron donor for the generation of $\Delta\bar{\mu}_{H^+}$, even though the vesicles respire at higher rates in the presence of other substrates (Barnes & Kaback, 1971; Schuldiner & Kaback, 1975; Stroobant & Kaback, 1975; Ramos et al., 1976).

Incubation of *E. coli* vesicles with radioactive D-lactate results in stoichiometric conversion to pyruvate (Kaback & Milner, 1970; Barnes & Kaback, 1970), and the D-lactate dehydrogenase (D-LDH) that catalyzes this reaction has been solubilized from the membrane and purified to homogeneity (Kohn & Kaback, 1973; Futai, 1973; Pratt et al., 1979). The enzyme has a molecular weight of 70K ± 10%, is composed of a single polypeptide chain containing 1 mol of flavin adenine dinucleotide (FAD) per mol of protein, exhibits a high degree of specificity for D(−)-lactate, and is specifically inactivated by the "suicide substrate" 2-hydroxy-3-butynoic acid (HBA) (Walsh et al., 1972). Moreover, antibody inhibition (Short et al., 1975a,b) and immunoadsorption studies (Owen & Kaback, 1978, 1979a,b) demonstrate clearly that the protein is associated with the cytoplasmic surface of the plasma membrane.

Vesicles prepared from mutants specifically defective in D-LDH do not catalyze D-lactate oxidation or D-lactate-dependent active transport (Hong & Kaback, 1972). However, the vesicles can be reconstituted with D-LDH by exposure to crude (Reeves et al., 1973) or purified (Short et al., 1974) enzyme preparations, and in the reconstituted system, it is apparent that the enzyme is bound to the outer surface of the vesicle membrane (Short et al., 1975a,b; Futai, 1975). Remarkably, despite the abnormal location of the enzyme, oxidation of D-lactate by reconstituted *dld*⁻ vesicles leads to the generation of a $\Delta\bar{\mu}_{H^+}$ that is indistinguishable in polarity and

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[1] Abbreviations: $\Delta\bar{\mu}_{H^+}$, electrochemical gradient of protons; D-LDH, D-lactate dehydrogenase; FAD, flavin adenine dinucleotide; HBA, 2-hydroxy-3-butynoic acid; NDH, NADH dehydrogenase; Cl_3CCOOH, trichloroacetic acid; PBS, phosphate-buffered saline; $NaDodSO_4$, sodium dodecyl sulfate; PMS, phenazine methosulfate; Tris, tris(hydroxymethyl)aminomethane.

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magnitude from that observed in native vesicles where D-LDH is bound to the opposite side of the membrane (Short et al., 1975b; S. Ramos and H. R. Kaback, unpublished observations; Olsiewski et al., 1981).

The techniques of molecular genetics allow cloning of specific DNA sequences, thus permitting the amplification of specific proteins, and it has become apparent over the past few years that these techniques are applicable to the bacterial plasma membrane (Raetz et al., 1977; Teather et al., 1978; Tyach et al., 1979; Creeger & Rothfield, 1979; Creeger et al., 1979; Motojima et al., 1979; Hugenholtz et al., 1981). In the context of this paper, Young and his colleagues have cloned the genes encoding one of the NADH dehydrogenases (NDH; Young et al., 1978; Jaworowski et al., 1981a,b) and D-LDH (Young et al., 1982) in *E. coli* and succeeded in amplifying the levels of each enzyme significantly. In addition, the *ndh* gene has been sequenced and the amino acid sequence of its product deduced from the nucleotide sequence of the gene (Young et al., 1981). Finally, catalytically active NDH has been synthesized in vitro by using a crude transcription/translation system (Poulis et al., 1981).

In this paper, we describe the plasmid-directed synthesis of D-LDH in minicells and the in vitro synthesis of the functional enzyme in a more defined transcription/translation system. Interestingly, the in vitro synthesis of the enzyme is stimulated by its cofactor. Moreover, evidence is presented indicating that this protein is synthesized in mature form and becomes membrane bound without a leader sequence. In another paper,[2] the effects of amplifying D-LDH and NDH on the bioenergetics of active transport in isolated membrane vesicles are described.

Materials and Methods

Organisms. *E. coli* IY36 is a K-12 derivative defective in NDH (Young & Wallace, 1976), and *E. coli* IY83 is derived from *E. coli* ML 308-225*dld*-3 (Hong & Kaback, 1972). Both strains carry the plasmid pIY2 which contains an insert encoding D-LDH (Young et al., 1982). The minicell-producing strain *E. coli* X1411 (Frazer & Curtiss, 1975) was generously provided by Dr. R. Curtiss. Unless specified otherwise, IY36 and IY83 were grown in Luria broth (Lennox, 1955) supplemented with ampicillin (25 μg/mL) at 37 °C on a rotary shaker. The transformed minicell-producing strain (X1411-36/20; *E. coli* X1411 transformed with pIY2) was grown in a minimal salts medium (Stroobant et al., 1972) supplemented with 30 mM mannitol, 0.1% yeast extract, 0.1% casamino acids, 1 μM thiamine hydrochloride, and 25 μg/mL ampicillin. For labeling experiments, yeast extract and casamino acids were omitted from the medium.

Minicells were purified from late-exponential cultures by a series of low-speed centrifugations (800*g* 5 times), followed by a minimum of three passages of the supernatant through 1.5-μm pore size nitrocellulose filters (Millipore). After each passage, the filtrate was assessed for intact cells by phase-contrast microscopy. The final filtrate was centrifuged at 5000*g* for 30 min; the pellet was over 98% minicells as judged by phase-contrast microscopy.

Plasmid Isolation and Purification. Plasmid DNA was amplified by using chloramphenicol (Clewell, 1972), and supercoiled DNA was purified either by acidic phenol extraction (Zasloff et al., 1978) or by a modification (Maeda, 1981) of the cleared lysate method of Clewell & Helinski (1969). The purity of the DNA was assayed by agarose electrophoresis (Southern, 1975). In some of the preparations used for in vitro protein synthesis, RNase treatment was omitted, as residual RNase activity inhibited the system.

Transformation. Transformation was affected by a modification (Santos & Kaback, 1981) of the method of Wensink et al. (1974).

Labeling of Minicells. Purified minicells were resuspended in mineral salts (Stroobant et al., 1972) supplemented with 30 mM mannitol, 1 μM thiamine hydrochloride, and 25 μg/mL ampicillin to an optical density of 10 at 600 nm. An aliquot (30 μL) of the suspension was added to 10 μL of an amino acid mixture (9 mM each with methionine omitted), and the mixture was incubated at 37 °C for 30 min. At this time, 5 μL of [^{35}S]methionine (500 μCi/mmol) was added, and the incubation was continued for a given period of time at 37 °C. Labeled minicells were then washed thoroughly by repeated centrifugation at 20000*g* for 15 min and resuspended to an appropriate concentration as described. The suspensions were then frozen and thawed 5 times and sonicated for 4 min (eight 30-s periods of sonication interspersed with 1-min cooling periods) prior to preparation for electrophoresis or immunoprecipitation.

DNA-Directed in Vitro Protein Synthesis. In vitro protein synthesis was performed with given DNAs as template as described by Kung et al. (1975). The system contained (in 35-μL final volume, unless noted otherwise) 15 mM Tris–acetate (pH 8.2), 11 mM sodium dimethylglutarate, 35 mM ammonium acetate, 65 mM potassium acetate, 10 mM magnesium acetate, 0.8 mM spermidine hydrochloride, 2.4 mM dithiothreitol, 0.93 mM each of UTP, CTP, and GTP, 3.0 mM ATP, 24 mM phosphoenolpyruvate, 0.2 μg of pyruvate kinase, 0.05 mM ppGpp, 0.7 mM 3′,5′-cAMP, 15 μM $N^{5,10}$-methenyltetrahydrofolate, 12.5 μg of *E. coli* tRNA, 0.63 mg of poly(ethylene glycol) 6000, approximately 6 μg of plasmid DNA, 0.4 mM isopropyl 1-thio-β-D-galactopyranoside, 0.112 mM each of every amino acid, 0.6 A_{260} unit of ammonium chloride washed ribosomes, a 0.25 M DEAE salt eluate (110 μg of protein), and a 1 M DEAE salt eluate (7.5 μg of protein); methionine was replaced with carrier-free [^{35}S]-methionine (1.0 μCi) at a final concentration of 0.1 mM. In given experiments, purified RNA polymerase (2.5 μg) in the presence and absence of L factor (1 μg) was substituted for the 1 M DEAE salt eluate. Where indicated, samples were precipitated with hot trichloroacetic acid (Cl_3CCOOH) by adding a small aliquot (3 μL) of the incubation mixture to 2.0 mL of 10% Cl_3CCOOH followed by heating at 90 °C for 15 min and filtration through 0.45-μm pore size nitrocellulose filters (Millipore).

Preparation of D-LDH and Anti-D-LDH. D-LDH was purified and assayed as described (Kohn & Kaback, 1973; Kaczorowski et al., 1978), and antiserum against the purified enzyme was prepared according to Short et al. (1975a). IgG was purified by ammonium sulfate precipitation followed by DEAE chromatography (Harboe & Ingild, 1973). Immunoprecipitation was carried out by adding 150 μL of unfractionated antiserum or purified IgG to a mixture consisting of 35 μL of sample (from an in vitro protein synthesis reaction or disrupted minicells), 500 μL of phosphate-buffered saline (PBS; 0.15 M sodium phosphate and 0.15 m sodium chloride, pH 7.2), 50 μL of 10% Triton X-100, and 10 μL of purified D-LDH (30 μg of protein) added as carrier. Samples were incubated at 37 °C for 2 h and the immunoprecipitates washed 3 times with PBS.

Sodium Dodecyl Sulfate–Polyacrylamide Gel Electrophoresis. The procedure of Laemmli (1970) was used

[2] E. Santos, I. G. Young, P. Owen, and H. R. Kaback, unpublished experiments.

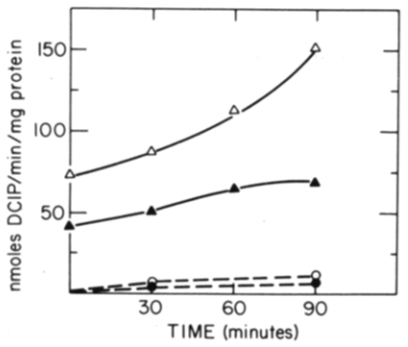

FIGURE 1: D-LDH activity in X1411 and X1411-36/20 minicells. Minicells purified as described under Materials and Methods were resuspended in minimal salts medium (Stroobant et al., 1972) supplemented with 30 mM mannitol, 0.1% yeast extract, 0.1% casamino acids, 1 μM thiamine hydrochloride, and 25 μg/mL ampicillin to a final concentration of 10^9 minicells per mL (ca. 8 mg of protein per mL) and incubated at 37 °C. At given times, samples (20 μL) were removed, washed twice with 0.1 M potassium phosphate (pH 7.2), and assayed for D-lactate-dependent dichlorophenolindophenol reduction (Kohn & Kaback, 1973; Kaczorowski et al., 1978). Where indicated, HBA was added to the minicell suspensions to a final concentration of 0.1 mM. (▲) X1411 minicells; (△) X1411-36/20 minicells; (●) X1411 minicells in the presence of HBA; (○) X1411-36/20 minicells in the presence of HBA.

throughout with 12% uniform polyacrylamide gels or 7.5–20% gradient gels in the separating phase. The stacking gel was 5% acrylamide. Samples were dissolved in sample buffer and boiled for 2 min before application to the gels. Electrophoresis was carried out either overnight at a constant voltage of 60 V or for 3–4 h at 30 mA/gel slab. Gels were stained with Coomassie brilliant blue, dried, and exposed for autoradiography on X-Omat R film (Kodak). Molecular weight standards used were the following: β-gal, β-galactosidase, 116K; BSA, bovine serum albumin, 68K; OAA, ovalbumin, 45K; DNase, deoxyribonuclease, 30K; chy, chymotrypsinogen, 25K; myo, myoglobin, 19K; and RNase, ribonuclease, 13.7K.

Detection of D-LDH Activity after in Vitro Synthesis. Conditions for in vitro synthesis were as described above except that the reactions were scaled up 5-fold (175-μL total volume), and Triton X-100 and FAD were added to final concentrations of 0.1% and 30 μg/mL, respectively. After incubation for a given period of time, lithium D-lactate and phenazine methosulfate (PMS)[3] were added to final concentrations of 35 and 0.1 mM, respectively, and the samples were incubated at room temperature overnight and lyophilized. Dried samples were derivatized with 2% methoxyamine (in pyridine) for 3 h at 60 °C to obtain the methoxine derivatives of keto groups, and then the trimethylsilyl derivatives were prepared by using *N,O*-bis(trimethylsilyl)trifluoroacetamide and 1% trimethylchlorosilane (Gardener & Horning, 1966). Samples were chromatographed in a Hewlett-Packard gas–liquid chromatograph (Model HP 5710A) equipped with a 1-m column consisting of a GCQ 100/120 support and a liquid phase of 3% OV-17. The run was programmed at a rate of 2 °C/min with an initial temperature of 80 °C and a final temperature of 260 °C. Lithium D-lactate and sodium pyruvate were used as standards.

Protein Determinations. Protein was measured according to Lowry et al. (1951) with bovine serum albumin as standard.

Results

Plasmid-Directed Synthesis of D-LDH in Minicells. The expression of D-LDH from pIY2 was first examined in minicells. As shown in Figure 1, X1411-36/20 minicells exhibit a higher basal level of D-LDH activity than minicells from the untransformed parental strain, and the activity increases about 2-fold during incubation in rich medium. In contrast, the D-LDH activity of the parental minicells increases by only about 20% under the same conditions. Importantly, moreover, in both cases, D-LDH activity is completely abolished when the minicells are treated with HBA, a highly specific suicide substrate for D-LDH (Walsh et al., 1972).

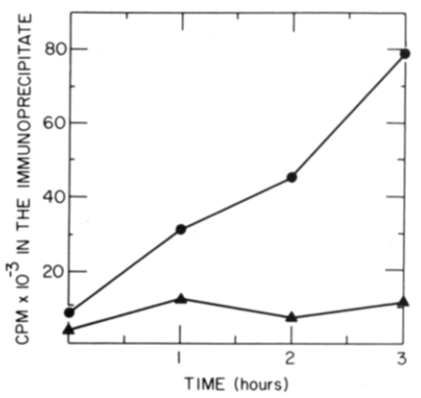

FIGURE 2: Incorporation of radioactivity into anti-D-LDH immunoprecipitates from X1411 (▲) and X1411-36/20 (●) minicells. Minicells were incubated with [^{35}S]methionine for indicated periods of time, treated as described under Materials and Methods, and immunoprecipitated with anti-D-LDH antiserum. The immunoprecipitates were washed in PBS, dissolved in 1.5% $NaDodSO_4$, and assayed for radioactivity.

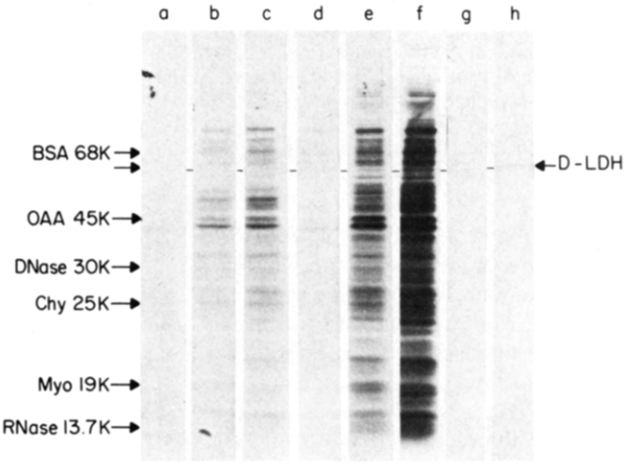

FIGURE 3: $NaDodSO_4$–polyacrylamide gel electrophoresis of labeled X1411 and X1411-36/20 minicells. Minicells of X1411 and X1411-36/20 were purified, labeled with [^{35}S]methionine, processed, and subjected to $NaDodSO_4$–polyacrylamide gel electrophoresis and radioautography as described under Materials and Methods. Lanes a, b, and c show X1411 minicells incubated with [^{35}S]methionine for 0, 40, and 90 min, respectively; lanes d, e, and f show X1411-36/20 incubated with [^{35}S]methionine for 0, 40, and 90 min, respectively; lanes g and h are X1411 and X1411-36/20 minicells, respectively, incubated with [^{35}S]methionine for 40 min, broken, and immunoprecipitated with anti-D-LDH IgG. Molecular weight standards and the position of purified D-LDH are provided. The separating gel was a gradient of 7.5–20% acrylamide.

When a similar experiment is carried out in the presence of [^{35}S]methionine, followed by disruption of the minicells and immunoprecipitation with antiserum prepared against purified D-LDH, the level of radioactivity in the immunoprecipitate from X1411-36/20 increases with time, while the radioactivity in the analogous immunoprecipitate from the parental minicells remains constant (Figure 2). Finally, it is apparent from the radioautographs presented in Figure 3 that there is time-dependent incorporation of [^{35}S]methionine into a protein that

[3] Phenazine methosulfate is autoxidizable, thus ensuring turnover of D-LDH.

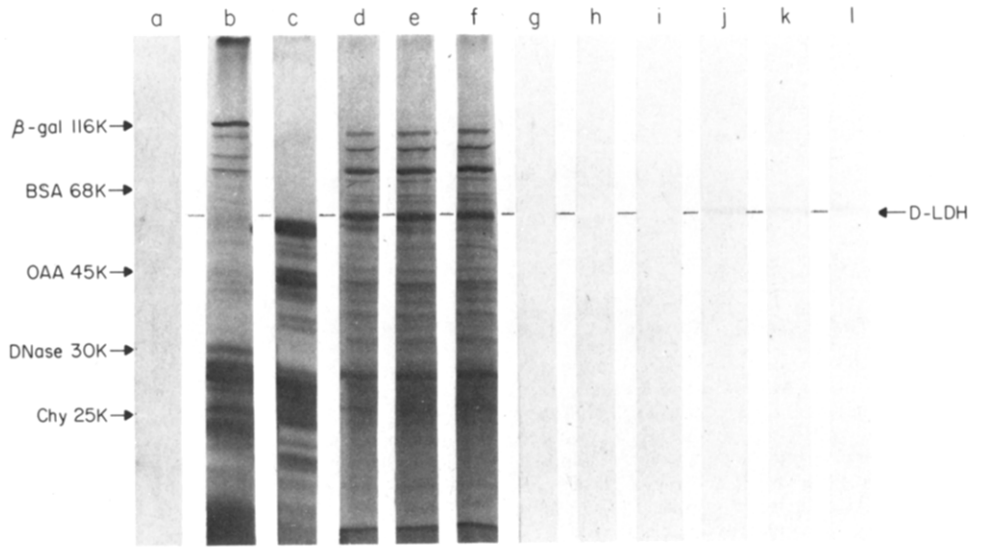

FIGURE 4: pIY2-directed in vitro synthesis of D-LDH. In vitro protein synthesis was carried out in the presence of [^{35}S]methionine by using the fractionated system of Kung et al. (1975) with given DNAs as templates (cf. below) as described under Materials and Methods. Samples of the reaction mixtures were analyzed by $NaDodSO_4$–polyacrylamide gel electrophoresis and radioautographed either directly (lanes a–f) or after immunoprecipitation with anti-D-LDH IgG (lanes g–l). Positions of given molecular weight standards are provided at the left, and the position of purified D-LDH is indicated at the right. Samples analyzed in each lane were as follows: (a) complete reaction mixture, no DNA; (b) complete reaction mixture, ps*lac* template; (c) complete reaction mixture, pGM706 template; (d) complete reaction mixture, pIY2 template; (e) complete reaction mixture, pIY2 template plus 0.14 mM 2,4-dinitrophenol; (f) complete reaction mixture, pIY2 template plus 1.4 mM lidocaine. Lanes g–l contain corresponding anti-D-LDH IgG immunoprecipitates from the same reaction mixtures in the same order. The separating gel was 12% acrylamide.

coelectrophoreses with authentic D-LDH during $NaDodSO_4$–polyacrylamide gel electrophoresis (lanes d–f) and that this protein represents the only radioactive band present after immunoprecipitation with anti-D-LDH IgG (lane h). No such increase in radioactivity is observed in the band corresponding to D-LDH in analogous experiments carried out with parental minicells (lanes a–c) or with minicells transformed with pGM706 (the vector for *dld*) or pIY1 (pGM706 containing the structural gene for NDH; not shown). Furthermore, no radioactive band is apparent after immunoprecipitation of the control minicell extracts (lane g).

It is clear from these experiments that pIY2 carries the structural gene for D-LDH and that the plasmid can direct the synthesis of this protein in vivo. It is also noteworthy in this context that transformation of *E. coli dld*$^-$ strains with pIY2 causes the mutants to regain the ability to grow on D-lactate and vesicles from these strains to regain the capacity for D-lactate-dependent active transport.[2]

DNA-Directed in Vitro Synthesis of D-LDH. In order to study cell-free synthesis of D-LDH, the in vitro transcription/translation system defined by Kung et al. (1975) was utilized. The data presented in Table I represent typical results of experiments in which the incorporation of [^{35}S]methionine into hot Cl_3CCOOH precipitates and into material precipitated by purified anti-D-LDH IgG was measured. In the absence of DNA, little incorporation of radioactivity into material precipitated by either means is observed, and incorporation into the hot Cl_3CCOOH precipitates increases markedly when pIY2, pGM706, or ps*lac* are employed as templates. With pIY2 as template, moreover, about 23% of the [^{35}S]methionine incorporated into the hot Cl_3CCOOH precipitates is immunoprecipitated with anti-D-LDH IgG, as compared to only 7–8% with pGM706 or ps*lac* as templates. In addition, when FAD is added to the reaction mixtures, incorporation of [^{35}S]methionine into pIY2-directed immunoprecipitable material increases about 2-fold, while no significant increase is observed in the presence of FAD with ps*lac*. Although not shown, essentially identical results were obtained when [^{14}C]leucine was used in place of [^{35}S]methionine.

Table I: Incorporation of [^{35}S]Methionine into Protein Synthesized in Vitro[a]

template DNA	cpm × 10^{-3}/reaction mixture: total incubation	cpm × 10^{-3}/reaction mixture: material precipitated by anti-D-LDH IgG
none	237	7 (3)
pIY2		
−FAD	2508	585 (23)
+FAD	2098	943 (45)
pGM706		
−FAD	2100	168 (8)
+FAD	nd	nd
ps*lac*		
−FAD	2897	231 (8)
+FAD	2438	170 (7)

[a] Incubations were carried out as described under Materials and Methods with given DNAs as template in a final volume of 70 μL. Where indicated, 2.7 μg of FAD was added to the samples. Total incorporation was determined by hot Cl_3CCOOH precipitation, and incorporation into immunoprecipitates was determined with IgG prepared against D-LDH as described under Materials and Methods. The values given in parentheses represent the percentage of the total radioactivity incorporated found in the immunoprecipitates. The specific activity of the [^{35}S]methionine was 500 Ci/mmol.

When the reaction mixtures described in Table I are subjected to $NaDodSO_4$–polyacrylamide gel electrophoresis, discrete translation products are readily visualized by radioautography (Figure 4). In control samples from which template DNA was omitted, no radioactive bands are observed when the total reaction mixture is electrophoresed (lane a), and accordingly, no radioactive bands are observed in the analogous immunoprecipitate (lane g). Similarly, although labeled products are observed with ps*lac*- and pGM706-directed reaction mixtures (lanes b and c), no radioactive material is observed at a position corresponding to that of purified D-LDH, and furthermore, all of the products encoded by these

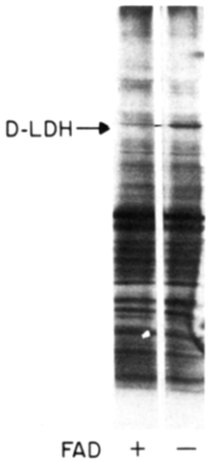

FIGURE 5: Effect of FAD on the expression of the *dld* gene. Synthesis of D-LDH was carried out in vitro with RNase-treated pIY2 DNA in the presence of [^{35}S]methionine as described under Materials and Methods. As indicated, the reactions were carried out in the absence or presence of FAD at a final concentration of 20 μg/mL. Samples were subjected to NaDodSO$_4$–polyacrylamide gel electrophoresis, and radioautography was as described under Materials and Methods. The separating gel was a 7.5–20% acrylamide gradient.

DNAs are completely absent after immunoprecipitaiton (lanes h and i). Electrophoresis of reaction mixtures containing pIY2 DNA reveals a number of radioactive products (lane f), only one of which is precipitated by anti-D-LDH IgG (lane l), and importantly, this band exhibits an electrophoretic mobility identical with that of authentic D-LDH. It is also noteworthy that addition of 2,4-dinitrophenol (Date et al., 1980) or lidocaine (Gaida et al., 1979), which reportedly inhibits leader peptidase activity, has no effect on the pattern obtained with the entire reaction mixture (lanes d and e) or the immunoprecipitated material (lanes j and k). Finally, the synthesis of the band corresponding to D-LDH is enhanced when FAD is present in the reaction mixtures (Figure 5). These data, taken as a whole, provide a strong indication that D-LDH is not synthesized in precursor form and that the expression of the *dld* gene is stimulated by the presence of FAD.

Another interesting observation concerning the expression of the *dld* gene is the finding that the L factor (Kung et al., 1975) inhibits the in vitro synthesis of D-LDH (Figure 6, compare − and + lanes for pIY2). In contrast, the expression of other genes on pIY2 appears to be unaffected by the L factor (cf. the band at ca. 29K) or enhanced (cf. the faint band at ca. 70K), while the synthesis of a number of proteins encoded by pGM706 and the 46K protein (NDH) encoded by the *ndh* gene on pIY1 (Young et al., 1978, 1981; Jaworowski et al., 1981a,b; Poulis et al., 1981) is enhanced by the L factor. This inhibitory effect of the L factor on the expression of *dld* is noteworthy because the factor was postulated originally to enhance the synthesis of translation products larger than 30K by inhibiting premature termination (Kung et al., 1975).

Activity of D-*LDH Synthesized in Vitro.* Since D-LDH contains tightly bound FAD as a prosthetic group (Kohn & Kaback, 1973; Futai, 1973), and the cofactor enhances the in vitro synthesis of the product encoded by the *dld* gene, it seemed likely that the translation product might be enzymatically active. Although enhanced rates of D-lactate-dependent dichlorophenolindophenol reduction are observed with in vitro reaction mixtures containing pIY2 relative to reaction mixtures containing control plasmids, the reaction rates are very low presumably because of the relatively low catalytic turnover of the enzyme and because very little protein is synthesized. For these reasons, the in vitro reaction mixtures

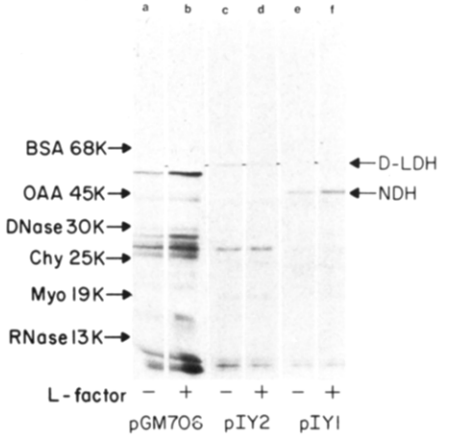

FIGURE 6: Effect of L factor on the expression of plasmids pGM706, pIY1, and pIY2. RNase-treated preparations of plasmid DNA were used as templates for in vitro protein synthesis in the absence or in the presence of 1 μg of L factor per reaction mixture as described under Materials and Methods. The samples were then analyzed directly by NaDodSO$_4$–polyacrylamide gel electrophoresis and radioautography. Molecular weight standards and the positions of D-LDH and NDH are provided. Samples analyzed in each lane were as follows: (lanes a and b) pGM706 template minus or plus L factor, respectively; (lanes c and d) pIY2 template minus or plus L factor, respectively; (lanes e and f) pIY1 template minus or plus L factor, respectively. The separating gel was a 7.5–20% acrylamide gradient.

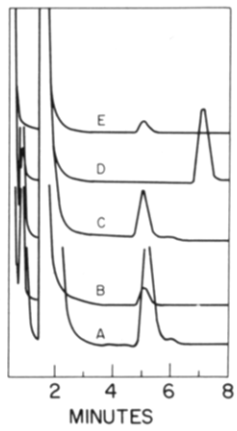

FIGURE 7: Utilization of D-lactate by D-LDH synthesized in vitro. In vitro protein synthesis was carried out for 90 min in a final valume of 175 μL in the presence of FAD (30 μg/mL) and Triton X-100 (0.1%), and the reaction mixtures were then incubated with D-lactate in the presence of PMS as described under Materials and Methods. The samples were lyophilized, and the methoxime and trimethylsilyl derivatives were prepared and analyzed by gas–liquid chromatography as described under Materials and Methods. Lines D and E are standards (400 μg of sodium pyruvate and 50 μg of lithium D-lactate, respectively). Line A corresponds to an incubation with 15 μg of ps*lac* DNA. Lines B and C correspond to incubations with 50 and 25 μg of pIY2 DNA, respectively. Integration of the areas under the D-lactate peak yields values of 500, 68, and 170 μg for lines A, B, and C, respectively.

were scaled up 5-fold, and Triton X-100 was added in order to keep the protein from aggregating (Kaczorowski et al., 1978). Protein synthesis was allowed to proceed for 90 min, D-lactate was added, and the reaction mixtures were incubated for a prolonged period of time under conditions designed to ensure enzyme turnover (cf. Materials and Methods).[3] Subsequently, the samples were subjected to trimethylsilylation followed by gas–liquid chromatography (Figure 7). As shown, no utilization of D-lactate is observed in control reaction mixtures using ps*lac* as template (A), while in the pIY2-directed reaction mixtures, there is clear utilization of substrate (B). Moreover, the quantity of D-lactate utilized appears to be related to the amount of pIY2 DNA present in the reaction

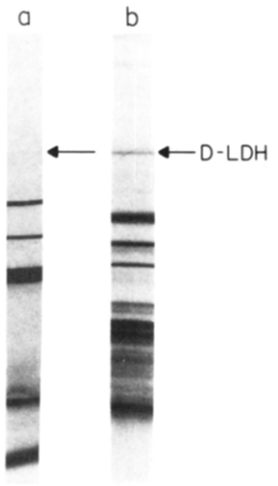

FIGURE 8: Binding of D-LDH synthesized in vitro to membrane vesicles. In vitro protein synthesis was carried out in a total volume of 35 μL for 90 min as described under Materials and Methods. The reaction mixtures contained 6.3 μg of plasmid pIY2 and, in addition, 10 μg of inverted *E. coli* ML 308–225 membrane vesicle protein (Reenstra et al., 1980). The reaction mixture was then centrifuged for 1 h at 130000g and the supernatant aspirated. An aliquot (10 μL) of 4-fold concentrated sample buffer was added to the supernatant, and 25 μL of the mixture was applied to lane a. The pellet was resuspended and washed once in 50 mM sodium phosphate (pH 7.2), dissolved in 15 μL of 2-fold concentrated sample buffer, and applied to lane b. The position of purified D-LDH is shown on the right.

mixtures (compare lines B and C). Although radioactive pyruvate is not detected in these studies, this is not surprising since the reaction mixtures contain pyruvate kinase and phosphoenolpyruvate as an ATP-generating system.

Binding of Newly Synthesized D-*LDH to the Membrane.* Addition of bacterial membrane vesicles or, particularly, liposomes to the transcription/translation system results in marked inhibition of in vitro protein synthesis, but it is apparent from experiments that will not be presented that inhibition can be overcome to a great extent by increasing the concentration of initiation factor Tu. Thus, in the following experiments (Figure 8), where the in vitro synthesis of D-LDH was carried out in the presence of inverted *E. coli* membrane vesicles (Reenstra et al., 1980), the activity of the system (as judged by the synthesis of both β-galactosidase with ps*lac* as template and D-LDH with pIY2 as template) was at least 40% that of the control samples incubated in the absence of vesicles. The reactions were performed with FAD and in the presence of inverted membrane vesicles, and after protein synthesis was allowed to proceed, the samples were fractionated by high-speed centrifugation, and the supernatant and membrane fractions were subjected to $NaDodSO_4$–polyacrylamide gel electrophoresis (lanes a and b, respectively). Clearly, the band corresponding to purified D-LDH partitions almost exclusively into the membrane fraction and is essentially absent from the supernatant.

Discussion

These results provide a clear demonstration that expression of the *dld* gene carried on the recombinant plasmid pIY2 (Young et al., 1982) can be achieved either in vivo by utilizing transformed minicells or in vitro with a fractionated DNA-directed protein synthesis system (Kung et al., 1975). The use of both systems in conjunction provides a strong indication that D-LDH, like NDH (Poulis et al., 1981), is synthesized in mature form and binds to the membrane in the absence of posttranslational proteolytic modification. Thus, only one product is observed after immunoprecipitation with antibody directed against purified D-LDH, and the appearance of the product is not altered by conditions that are thought to inhibit leader peptidase activity in *E. coli* membranes. Furthermore, the product corresponding to mature D-LDH appears to partition spontaneously into the membrane during or after synthesis, an observation that is not surprising given previous observations demonstrating that the purified enzyme readily associates with the membrane in a functional manner (Reeves et al., 1973; Short et al., 1974, 1975a,b; Futai, 1975; Olsiewski et al., 1981).

One unique and important observation resulting from the use of a fractionated transcription/translation system concerns the effect of FAD, the prosthetic group for D-LDH, on the synthesis of the enzyme. As judged by immunoprecipitation of the in vitro reaction mixtures and by $NaDodSO_4$–polyacrylamide gel electrophoresis of the immunoprecipitated material, there is a 2-fold increase in the synthesis of D-LDH in the presence of FAD. Although it cannot be ascertained from the present experiments whether the coenzyme enhances synthesis at the transcriptional or translational level, it is apparent that other membrane-bound enzymes may be subject to similar means of regulation. Induction of formate dehydrogenase and nitrate reductase in anaerobic *E. coli*, for example, requires the presence of selenite and molybdate, respectively, in addition to formate and nitrate (Lester & De Moss, 1971).

Another interesting and unexpected aspect of the in vitro experiments is the effect of L factor on the synthesis of D-LDH. Contrary to previous findings demonstrating that L factor stimulates the in vitro synthesis of β-galactosidase (Kung et al., 1975) and the ββ′ subunit of RNA polymerase (Schultz et al., 1979), L factor inhibits the expression of the *dld* gene. On the other hand, in agreement with the earlier observations, L factor stimulates expression of the *ndh* gene cloned in plasmid pIY1. Recently, L factor has been identified as the product of the *nusA* gene in *E. coli* which interacts with bacteriophage λ N gene protein and prevents premature termination of transcription from early λ promoters (Greenblatt et al., 1980). The observations presented here (i.e., stimulation of expression of *ndh* and inhibition of *dld*) indicate that the role of L factor in transcriptional regulation may be more subtle, as suggested recently by Kassavetis & Chamberlin (1981).

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References

Barnes, E. M., & Kaback, H. R. (1970) *Proc. Natl. Acad. Sci. U.S.A. 66*, 1190.

Barnes, E. M., & Kaback, H. R. (1971) *J. Biol. Chem. 246*, 5518.

Clewell, D. B. (1972) *J. Bacteriol. 110*, 667.

Clewell, D. B., & Helinski, D. R. (1969) *Proc. Natl. Acad. Sci. U.S.A. 62*, 1159.

Creeger, E. S., & Rothfield, L. I. (1979) *J. Biol. Chem. 254*, 804.

Creeger, E. S., Chen, J. F., & Rothfield, L. I. (1979) *J. Biol. Chem. 254*, 811.

Date, T., Zwizinski, C., Ludmerer, S., & Wickner, W. (1980) *Proc. Natl. Acad. Sci. U.S.A. 77*, 827.

Frazer, A. C., & Curtiss, R., III (1975) *Curr. Top. Microbiol. Immunol. 69*, 3.

Futai, M. (1973) *Biochemistry 12*, 2468.
Futai, M. (1975) *J. Bacteriol. 124*, 470.
Gaida, R. E., Henderson, G. W., & Markovitz, A. (1979) *Proc. Natl. Acad. Sci. U.S.A. 76*, 2138.
Gardener, W. L., & Horning, E. C. (1966) *Biochim. Biophys. Acta 115*, 425.
Greenblatt, J., Li, J., Adhya, S., Friedman, D. I., Baron, L. S., Redfield, B., Kung, H., & Weissbach, H. (1980) *Proc. Natl. Acad. Sci. U.S.A. 77*, 1991.
Harboe, N., & Ingild, A. (1973) *Scand. J. Immunol. 2*, 161.
Harold, F. (1977) *Curr. Top. Bioenerg. 6*, 83.
Hong, J., & Kaback, H. R. (1972) *Proc. Natl. Acad. Sci. U.S.A. 69*, 3336.
Hugenholtz, J., Hong, J., & Kaback, H. R. (1981) *Proc. Natl. Acad. Sci. U.S.A. 78*, 3446.
Jaworowski, A., Campbell, H. D., Poulis, M. I., & Young, I. G. (1981a) *Biochemistry 20*, 2041.
Jaworowski, A., Mayo, G., Shaw, D. C., Campbell, H. D., & Young, I. (1981b) *Biochemistry 20*, 3621.
Kaback, H. R. (1972) *Biochim. Biophys. Acta 265*, 367.
Kaback, H. R. (1974) *Science (Washington, D.C.) 186*, 882.
Kaback, H. R. (1976) *J. Cell. Physiol. 89*, 575.
Kaback, H. R., & Milner, L. S. (1970) *Proc. Natl. Acad. Sci. U.S.A. 66*, 1108.
Kaczorowski, G., Kohn, L. D., & Kaback, H. R. (1978) *Methods Enzymol. 53*, 519.
Kassavetis, G. A., & Chamberlin, M. J. (1981) *J. Biol. Chem. 256*, 2777.
Kohn, L. D., & Kaback, H. R. (1973) *J. Biol. Chem. 248*, 7012.
Konings, W., & Boonstra, J. (1977) *Curr. Top. Membr. Transp. 9*, 177.
Kung, H., Spears, C., & Weissbach, H. (1975) *J. Biol. Chem. 250*, 1556.
Laemmli, V. K. (1970) *Nature (London) 227*, 680.
Lennox, E. S. (1955) *Virology 1*, 190.
Lester, R. L., & De Moss, J. A. (1971) *J. Bacteriol. 105*, 1006.
Lowry, D. H., Rosebrough, N. J., Farr, A. L., & Randall, R. J. (1951) *J. Biol. Chem. 193*, 265.
Maeda, S. (1981) *Methods Enzymol. 79*, 607.
Mitchell, P. (1963) *Biochem. Soc. Symp. No. 22*, 142.
Mitchell, P. (1968) *Chemiosmotic Coupling in Oxidative and Photosynthetic Phosphorylation*, Glynn Research, Ltd., Bodmin, England.
Mitchell, P. (1973) *J. Bioenerg. 4*, 63.
Motojima, K., Yamato, I., Anraku, Y., Nishimura, A., & Hirata, Y. (1979) *Proc. Natl. Acad. Sci. U.S.A. 76*, 6255.
Olsiewski, P., Kaczorowski, G., Walsh, C. T., & Kaback, H. R. (1981) *Biochemistry 20*, 6272.
Owen, P., & Kaback, H. R. (1978) *Proc. Natl. Acad. Sci. U.S.A. 75*, 3148.
Owen, P., & Kaback, H. R. (1979a) *Biochemistry 18*, 1413.
Owen, P., & Kaback, H. R. (1979b) *Biochemistry 18*, 1422.
Poulis, M. I., Shaw, D. C., Campbell, H. D., & Young, I. G. (1981) *Biochemistry 20*, 4178.
Pratt, E. A., Fung, L. W. M., Flowers, J. A., & Ho, C. (1979) *Biochemistry 18*, 312.
Raetz, C. R. H., Larson, T. J., & Dowhan, W. (1977) *Proc. Natl. Acad. Sci. U.S.A. 74*, 1412.
Ramos, S., Schuldiner, S., & Kaback, H. R. (1976) *Proc. Natl. Acad. Sci. U.S.A. 73*, 1892.
Reenstra, W. W., Patel, L., Rottenberg, H., & Kaback, H. R. (1980) *Biochemistry 19*, 1.
Reeves, J. P., Hong, J., & Kaback, H. R. (1973) *Proc. Natl. Acad. Sci. U.S.A. 70*, 1917.
Santos, E., & Kaback, H. R. (1981) *Biochem. Biophys. Res. Commun. 99*, 1153.
Schuldiner, S., & Kaback, H. R. (1975) *Biochemistry 14*, 5451.
Schultz, T. Z., Jerez, C., Goldberg, G., Kung, H., Huang, R. H., Brot, N., & Weissbach, H. (1979) *Proc. Natl. Acad. Sci. U.S.A. 76*, 6115.
Short, S. A., Kaback, H. R., & Kohn, L. D. (1974) *Proc. Natl. Acad. Sci. U.S.A. 71*, 1461.
Short, S. A., Kaback, H. R., Hawkins, T., & Kohn, L. D. (1975a) *J. Biol. Chem. 250*, 4285.
Short, S. A., Kaback, H. R., & Kohn, L. D. (1975b) *J. Biol. Chem. 250*, 4291.
Southern, E. M. (1975) *J. Mol. Biol. 98*, 503.
Stroobant, P., & Kaback, H. R. (1975) *Proc. Natl. Acad. Sci. U.S.A. 72*, 3970.
Stroobant, P., Young, I. G., & Gibson, F. (1972) *J. Bacteriol. 109*, 134.
Teather, R. M., Muller-Hill, B., Abrutsch, U., Aichele, G., & Overath, P. (1978) *Mol. Gen. Genet. 159*, 239.
Tyach, R. J., Hawrot, E., Satre, M., & Kennedy, E. (1979) *J. Biol. Chem. 254*, 627.
Walsh, C. T., Abeles, R. H., & Kaback, H. R. (1972) *J. Biol. Chem. 247*, 7858.
Wensink, P. C., Finnegan, D. J., Donelson, J. E., & Hogness, D. S. (1974) *Cell (Cambridge, Mass.) 3*, 315.
Young, I. G., & Wallace, B. J. (1976) *Biochim. Biophys. Acta 449*, 376.
Young, I. G., Jaworowski, A., & Poulis, M. I. (1978) *Gene 4*, 25.
Young, I. G., Rogers, B. L., Campbell, H. D., Jaworowski, A., & Shaw, D. C. (1981) *Eur. J. Biochem. 116*, 165.
Young, I. G., Jaworowski, A., & Poulis, M. (1982) *Biochemistry* (following paper in this issue).
Zasloff, M., Ginder, G. O., & Felsenfeld, G. (1978) *Nucleic Acids Res. 5*, 1139.