JOURNAL OF BACTERIOLOGY, June 1985, p. 1270–1275
0021-9193/85/061270-06$02.00/0

Vol. 162, No. 3

In Vitro and In Vivo Activation of L-Serine Deaminase in *Escherichia coli* K-12

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Received 30 November 1984/Accepted 26 February 1985

***Escherichia coli* L-serine deaminase (L-SD) in crude extracts made in glycylglycine could be activated by incubation with iron sulfate and dithiothreitol. This activation could also be demonstrated in vitro in two mutants which were physiologically deficient in L-SD activity in vivo. This suggests that these mutants were deficient not in L-SD but in an enzyme(s) activating L-SD. The suggestion is made that production of a functional L-SD in vivo requires activation of the structural gene product by an enzyme or enzymes that reduce the protein to an active form.**

An enzyme activity deaminating L-serine in *Escherichia coli* was described by Pardee and Prestidge in 1955 (24). They and subsequent investigators studied this activity mainly in permeabilized whole cells (23). Many factors which influence the synthesis of this activity are known (20). Mutants with more activity than the parent have been described previously (22), but other mutants showed very little activity in the permeabilized cell assay and were unable to deaminate L-serine in vivo (23).

It has proved much more difficult to assay the enzyme in cell extracts (21). In all bacteria studied, L-serine deaminase (L-SD) has an unstable activity, which may be protected and even activated by substrate and by ferrous ion (2, 6, 10, 19, 21). For instance, the enzyme purified from glycine-grown *Arthrobacter globiformis* was activated by preincubation with its substrate (10). Similarly, the *Clostridium acidiurici* enzyme was activated by incubation with Fe and dithiothreitol (DTT) (6), and a *Corynebacterium* enzyme showed unusual kinetics, suggesting that it too might be subject to activation (19).

The *E. coli* K-12 L-SD has been studied less. It also was shown to be very unstable in extracts (2). Like the *Clostridium* enzyme, it could be protected and activated with ferrous sulfate and DTT, as well as with its substrate and substrate analogs (21). However, the actual experiments were not readily reproducible, and the suggestion was made that the enzyme assayed in that study was essentially inactive (21).

V. K. Bankovskii recently informed us that *E. coli* K-12 L-SD could be purified in the presence of 1 M glycine (personal communication). Since we know that glycine interacts with the active site of the enzyme (21), we thought that glycylglycine might have the same stabilizing effects as glycine, without the inconvenience of competition with the substrate. We report here that the enzyme prepared in glycylglycine is quite stable and is subject to an elaborate activating system which is described in this paper. This activating system appears to be defective in two mutants previously described as being defective in L-SD (23).

MATERIALS AND METHODS

Cultures. The strains used, all derivatives of *E. coli* K-12, are described in Table 1. All experiments were done with strain MEW1 unless otherwise noted. The minimal medium (23) and the methods for making extracts (21) have been described previously. Where noted, cells were grown with excess iron by adding filter-sterilized ferrous sulfate to the growth medium at 30 mg/liter. The large amount of precipitate which formed was not removed. This excess of iron was added in case the original medium might be deficient in iron. However, the same results were obtained when cells were grown without added iron. Extracts were made either in 0.05 M glycylglycine (pH 8) or in 0.05 M potassium phosphate (pH 7.5), with 10^{-3} M $FeSO_4$ and 10^{-2} M DTT.

L-SD assays. The standard incubation mixture contained 50 μl of L-serine (20 mg/ml), 0 to 30 μl each of 10^{-3} M $FeSO_4$ and 10^{-2} M DTT, 5 to 50 μl of extract, and glycylglycine buffer to 210 μl. After incubation at 37°C for the times indicated, the reaction was stopped with dinitrophenylhydrazine in HCl and the keto acid was determined as previously described (21). Details of individual experiments are given in the text.

TABLE 1. Bacterial strains

Strain	Genotype and relevant characteristics	Source
CU1008	*ilvA*	L. S. Williams
MEW1	Δ*lac* derivative of CU1008	23
CAG5050	Mu::dX *C*am (Amp^r *lac*) *pro lac* (F′ *pro lacZ-8305*::Mucts62)	4
MEW128	Mutant (SGL$^-$) unable to grow with serine, glycine, and leucine; isolated by penicillin selection from strain CU1008	23
MEW191	SGL$^-$ derivative by Mu::dX insertion from CAG5050 into MEW1	23
MEW15	SGL$^-$ derivative by Mu::dX insertion from CAG5050 into MEW1	23
MEW206	Gluconeogenesis deficient by Mu::dX insertion from CAG5050 into MEW1	This work
CGSC 6092	*trxA*	B. Bachmann
A179	HfrC *trxA*::Tn*10*	25

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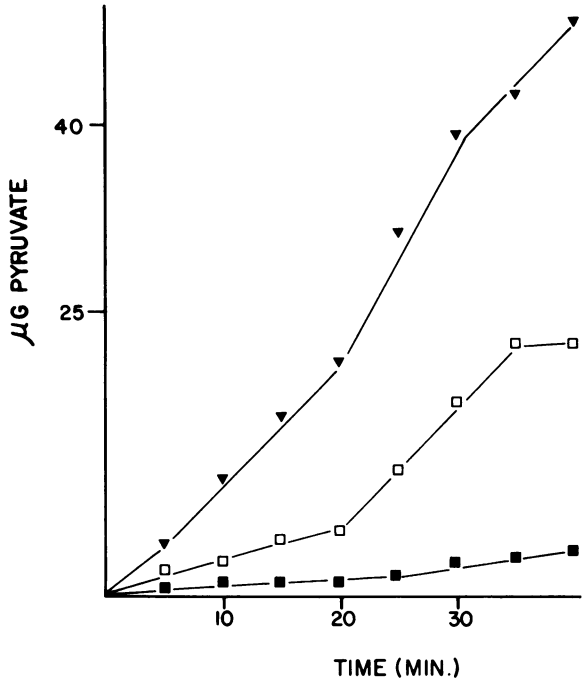

FIG. 1. Activation of L-SD by Fe and DTT. Samples (50 μl) of a crude extract (approximately 15 mg of protein per ml) in glycylglycine were incubated with the additions noted, and the amount of pyruvate formed as a function of time was determined. Additions to the assay were L-serine alone (■), plus 10^{-3} M Fe and 10^{-2} M DTT (□), or plus 3×10^{-3} M Fe and 3×10^{-2} M DTT (▼).

RESULTS

Stability of L-SD activity in glycylglycine buffer. Unlike extracts made in phosphate buffer (21), extracts in glycylglycine retained substantial activity for several days, whether frozen or at 4°C. L-SD activity could be precipitated with 50 to 60% ammonium sulfate, dissolved in glycylglycine buffer, and still remain stable for several days. However, very little activity could be recovered from Sephadex G100 columns run with 0.05 M glycylglycine, with or without Fe and DTT. A maximum of 15% of the activity put on the column could be recovered in the first fractions after the void volume. Attempts to reconstitute by pooling fractions were not successful.

Activation of L-SD activity in crude extracts. It was shown earlier that L-SD activity could be somewhat stabilized and occasionally activated by incubation with Fe and DTT (21). The preparation of extracts in glycylglycine buffer allowed us to describe the process of enzyme activation in detail. For these experiments, cells were grown in the usual medium with added $FeSO_4$, harvested, and extracted in glycylglycine with $FeSO_4$ and DTT. However, the same results were obtained when $FeSO_4$ was omitted from both growth medium and extract buffer.

Extracts made in this manner showed very little L-SD activity (Fig. 1, ■). However, when Fe and DTT were added to the assay mixture, all extracts deaminated L-serine at a more rapid rate from the start of the incubation (Fig. 1, □). With the higher concentrations of Fe and DTT (Fig. 1, ▼), the rate increased so rapidly that the rate between 25 and 30 min was only slightly faster than the rate in the first 5 min. The activation was much more obvious at the lower concentrations. In the experiment plotted in Fig. 1, □, the extract produced sixfold more pyruvate between 25 and 35 min of incubation than it did in the first 10 min. The details of the curves varied from experiment to experiment. In six extracts incubated with the lower concentrations of Fe and DTT, the final rate was two to six times faster than the initial rate.

The activation of the enzyme was seen most clearly in conditions which did not allow detectable catalytic activity. To show this, the extract was incubated with and without Fe and DTT for different lengths of time in the presence of a small amount of L-serine. After this preincubation period, a higher concentration of L-serine was added to start the catalytic reaction, and the amount of pyruvate produced was measured after 10 min. The amount of pyruvate produced in that 10-min incubation was greatly increased as a result of preincubation (Table 2). Preincubation therefore must have resulted in activation of L-SD.

Factors influencing activation. (i) Iron and DTT. To estimate the quantity of both factors actually needed, we grew

TABLE 3. Effect of variations in iron and DTT on L-SD activity[a]

DTT (mM)	L-SD activity at Fe concn (mM) of:			
	0	1	2	3
0	1.3	1.7	2.6	3.4
10	0.8	3.6	4.2	5.3
20	0.3	3.3	5.1	6.6
30	0.6	1.5	3.6	8.0

[a] Cells of strain MEW1 were grown in minimal medium and extracted in glycylglycine without iron or DTT. Assays were conducted in the presence of $FeSO_4$ or DTT, or both, at the molarities indicated. Activity is expressed in terms of nanomoles of pyruvate produced in a 10-min incubation with 20 μl of extract.

TABLE 2. Effect of preincubation on L-SD activity[a]

Length (min) of preincubation	L-SD activity (nmol of pyruvate formed) with:	
	No additions	Fe and DTT
0	2.9	4.85
5	3.2	4.85
9	3.4	4.85
14	3.8	6.9
20	3.6	10.5
25	3.8	13.7
29	4.8	15.7

[a] An extract of strain MEW1 made in glycylglycine buffer was incubated for the times indicated with 0.005 M serine, with and without 10^{-3} M Fe and 10^{-2} M DTT. After the preincubation periods noted, 0.05 M L-serine was added, and the amount of pyruvate formed in the next 10 min was determined.

TABLE 4. Effect of assay buffer on activation of L-SD[a]

Buffer	Fe and DTT	L-SD activity (nmol of pyruvate) after incubation for (min):			
		10	20	30	40
PO_4	−	21	48	50	83
PO_4	+	5	14	17	40
Glygly	−	29	44	56	82
Glygly	+	75	219	429	564

[a] An extract of strain MEW1 was made in 0.05 M phosphate buffer (pH 7.5). A 20-μl volume of extract was incubated for 10, 20, 30, and 40 min in a total volume of 260 μl, containing either 0.05 M PO_4 or 0.05 M glyclyglycine (Glygly) buffer, and 0.07 M L-serine in the presence and absence of 1.1×10^{-3}M $FeSO_4$ and 1.1×10^{-2} M DTT. The amount of pyruvate produced during the assay was determined. These are the results of one of four similar experiments.

TABLE 5. Effect of variations in preincubation conditions on L-SD activity[a]

Expt	Preincubation conditions	Addition to start reaction	LSD activity (nmol of pyruvate) after incubation for (min):			
			0	10	20	30
1	Fe, DTT, extract	L-serine	15	14	35	54
2	Fe, DTT, extract	L-serine	53	37	21	11
3	Fe, DTT	L-serine, extract	19	19	19	11
4	Fe, DTT, extract, 0.005 M L-serine	L-serine	22	22	67	118

[a] Activity is expressed as nanomoles of pyruvate produced in a 10-min incubation after a preincubation in glyclyglycine buffer for the times and conditions noted. L-Serine (50 mM) was added to start the reaction. Experiments 1, 3, and 4 were done with one extract, and experiment 2 was done with a different extract.

strain MEW1 in the usual medium without iron and made an extract in glycylglycine buffer without further additions. The assays then were made with various amounts of iron and DTT (Table 3).

Both factors were required for activation, and at surprisingly high concentrations. Fe alone only slightly activated the enzyme, and DTT alone actually inhibited the enzyme. Fe at a sufficiently high concentration overcame the DTT inhibition and activated the enzyme. Still higher amounts of Fe and DTT did not increase the activation and indeed became inhibitory.

Even when cells were grown in minimal medium to which excess iron had been added, and extracts were made in buffer containing both Fe and DTT, it was necessary to add both factors to the assay to fully activate the enzyme.

The data (Table 3) are the results of a single experiment. Since the kinetics of activation varied somewhat from experiment to experiment, results of an experiment based on one fixed time of assay also varied. However, although the experiments differed in detail, they generally were the same.

(ii) Possible substitutes for iron and DTT. To compare the effects of other compounds with those of Fe and DTT, we incubated the extract with 10^{-2} M DTT and each of the following compounds at 1.4×10^{-3} M: $MgSO_4$, $MnCl_2$, $CaSO_4$, K_2SO_4, $NiCl_2$, $NaMoO_4$, and $NaWO_4$. None of these either activated or inhibited the enzyme.

We similarly incubated the extract with 10^{-3} M $FeSO_4$ and each of the following at 10^{-2} M: mercaptoethanol, glutathionine, and sodium ascorbate. Only ascorbate activated the enzyme, but to a lesser extent than did DTT.

TABLE 6. Effect of dialysis on L-SD activity[a]

Expt	Fe and DTT added during:		L-SD activity (nmol of pyruvate/min per mg of protein)
	Dialysis	Assay	
1	+	−	6.3
2	+	+	33.7
3	−	−	1.5
4	−	+	20.7

[a] Extracts of strain MEW1 were made in glycylglycine buffer with $FeSO_4$ and dialyzed overnight against 0.01 M L-serine in glycylglycine buffer at 4°C with and without 10^{-4} M $FeSO_4$ and 10^{-2} M DTT. The dialyzed extracts were then assayed, with and without further additions of $FeSO_4$ and DTT. $FeSO_4$ and DTT were added to the assay in experiments 2 and 4 at 10^{-3} and 10^{-2} M, respectively. Since the extract was diluted only one-third for assaying, considerable iron was carried over into the assay in experiment 1.

TABLE 7. β-Galactosidase synthesis from insert *lacZ* in conditions known to induce L-SD[a]

Conditions	L-SD activity in strain MEW1 (mU)[b]	β-Galactosidase activity in strain:	
		MEW15	MEW191
No additions	19	44	461
Glycine and leucine added	110	69	490
1 h of UV	130	102	533
42°C	34	51	464
Luria broth	230	223	676

[a] The experiment was performed as described previously (23).
[b] 1 U = 1 nmol of Ketoacid produced by 0.01 ml of a 100-Klett Unit suspension of cells in 35 min.

(iii) Activation in phosphate buffer. To verify that our ability to demonstrate this activation was in fact due to the change in buffer, we made extracts in 0.05 M phosphate (pH 7.5) and assayed with and without iron and DTT in phosphate buffer and in glycylglycine. Some L-SD activity was seen when the extract was assayed in either buffer (Table 4). However, the activation with Fe and DTT could be demonstrated only in glycylglycine buffer. This clearly accounts for why activation by Fe and DTT could not be reproducibly shown in earlier work done in our laboratory.

(iv) Role of Fe, DTT, and serine during preincubation. One might suppose that during the preincubation period, Fe and DTT would react together and the product of that reaction would activate L-SD. This is most likely since iron and DTT

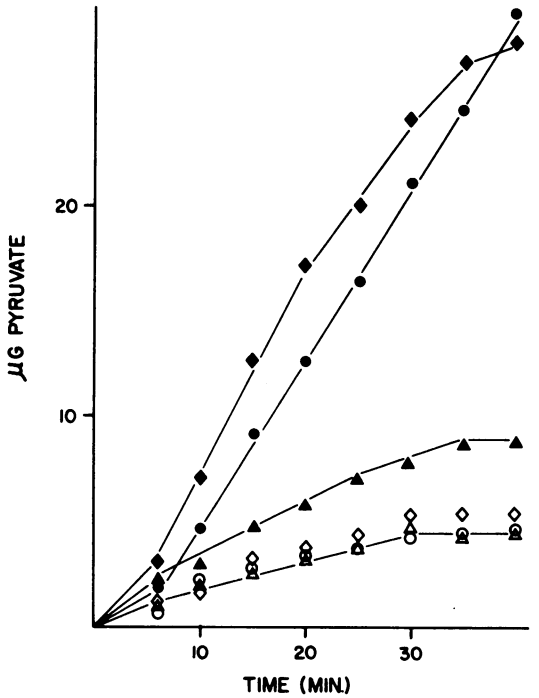

FIG. 2. Activation of L-SD in L-SD-deficient mutants. Samples (40 μl) of crude extracts (approximately 15 mg of protein per ml) of strain MEW 128 (◇, ◆), MEW 191 (○, ●), and MEW 15 (△, ▲) were incubated with (filled symbols) and without (empty symbols) 1.5×10^{-2} M Fe and 0.15 M DTT for the times indicated, and the amount of pyruvate formed was determined. For the values without effectors, the curves were almost identical, and only one is drawn.

could be shown to deaminate L-serine in glycylglycine buffer in a nonenzymatic reaction, although very slowly in comparison to the enzyme-catalyzed reaction (data not shown).

For Fe and DTT to activate, it was not sufficient to incubate them together in the absence of extract. When Fe and DTT were incubated for various lengths of time in glycylglycine buffer and then serine and extract were added, no activation was seen (Table 5, experiment 3). The extract indeed produced the same amount of pyruvate (19 nmol) before and after a 20-min incubation. The addition of L-serine along with iron and DTT (without extract) did not result in activation (data not shown).

This is consistent with two models. First, Fe and DTT might react together in a nonenzyme-catalyzed reaction, forming a transient intermediate; L-SD would be activated only if it were present at the time the intermediate were formed. Alternatively, the interaction of iron and DTT might itself be enzyme catalyzed.

With either model, L-serine might stabilize the enzyme and, at the low concentration provided, might have no other function. In fact, when serine was completely omitted from the preincubation mixture, results were quite variable. On some occasions, some activation was seen, although less than when serine was present (Table 5, experiments 1 and 4). On other occasions, activity was lost from the start (Table 5, experiment 2).

It seems likely then that Fe and DTT interacted with the L-SD protein, or with some other enzyme(s), in the extract, resulting in activation of L-SD. However, the L-SD protein was so unstable without L-serine that some substrate usually had to be added to preserve L-SD activity.

Reactivation of dialysates. To determine whether there were any cofactors other than Fe involved in the reaction, we dialyzed samples of the crude extract against glycylglycine with and without Fe and DTT (Table 6). The extract dialyzed in the absence of Fe and DTT showed no activity unless Fe and DTT were added to the assay; these additions were sufficient to restore activity (experiments 3 and 4). The extract dialyzed with Fe and DTT retained more activity (experiment 1) but was also activated by further addition (experiment 2). We conclude therefore that if the enzyme has any cofactors, they must be either tightly bound or replaceable by Fe and DTT.

Activation in L-SD-deficient mutants. Three mutants deficient in L-SD were isolated (23), two by insertion mutagenesis with Mu::dX (MEW15 and MEW191) and one by penicillin selection (MEW128). Since β-galactosidase activity in strain MEW15 was induced by the inducers of L-SD in strain MEW15 but not in strain MEW191 (Table 7), it seems likely that strain MEW15 carries an insertion in the structural gene for L-SD. Since both strains MEW191 and MEW128 required thiamine, it seems likely that they are similar and also different from strain MEW15 which was not thiamine requiring.

In physiological terms, all three mutants were deficient in L-SD activity in vivo. They were originally isolated as being unable to grow on plates supplemented with a combination of L-serine, glycine, and L-leucine as the carbon source (23). Moreover, they were unable to grow with L-serine as the nitrogen source (23). As judged by the standard whole-cell assay with toluene-treated cells, none of the three mutants produced L-SD activity in glucose minimal medium.

Although strains MEW191 and MEW128 were unable to deaminate L-serine in vivo, their deaminating system could be activated in vitro. When strains MEW191 and MEW128 were grown in minimal medium with Fe and extracts made with Fe and DTT, but assayed without additional Fe and DDT, they showed no activity (Fig. 2). However, extracts of both strains MEW191 and MEW128 could be markedly activated by incubation with Fe and DTT. This activation was somewhat slower than that seen in the parent strain, and the final activity reached was lower. Nonetheless, it is clear that these strains contained an activatable but nonfunctional form of L-SD.

The same experiment was performed with strain MEW15 (Fig. 2). It showed some activity with Fe and DTT but much less than did the other mutants. It may be surprising that strain MEW15 had any activity at all. However, it is clear that the other two mutants were defective in activation, and it is probable that strain MEW15 was defective in production of the L-SD protein.

Comparison of L-SD-deficient strains with known thioredoxin mutants. Phage T7 specifies a two-component DNA polymerase (13) consisting of the phage gene 5 product (a polymerase) and *E. coli* thioredoxin. Without the *E. coli* thioredoxin, the gene 5 product is inactive. Phage T7 therefore does not produce plaques on thioredoxin mutants (13). If any of our L-SD-deficient mutants synthesized a defective thioredoxin (or none at all), we would therefore expect them to be T7 resistant.

To test this, we plated approximately 2,000 phage T7 onto lawns of various strains of *E. coli*. The two thioredoxin mutants CGSC 6092 and A179 showed no plaques at all, whereas our parent strain MEW1 was completely lysed. Strain MEW128 showed about 100 barely visible plaques and was clearly much more resistant to T7 than was its parent, although somewhat less resistant than the strains known to be defective in thioredoxin.

Strain MEW191 was also resistant to T7, although somewhat less so. However, the situation with the insertion strains was more complex. Strain MEW191 produced 100 small irregular plaques, whereas strain MEW15 produced 100 larger plaques. One might consider from this that the mutations in both strains lead to T7 resistance. However we also studied strains carrying insertions of Mu::dX into two unrelated genes, strain DRN-1 and MEW206, which carry insertions in *serA* and in *pps* (data not shown). In both cases we saw about 300 large plaques, rather than the complete lysis seen in strain MEW1. It seems therefore that the Mu::dX insert itself hinders T7 propagation. Strain MEW191 probably has an additional component of resistance due to its mutation, but strain MEW15 does not. This is consistent with the idea that strain MEW15 carries an insert in the structural gene for L-SD.

DISCUSSION

We have shown here that the protein catalyzing L-serine deamination is not in its most active form in crude extracts of *E. coli* K-12. The rate of L-serine deamination increased gradually during a 40-min incubation with Fe and DTT. This activation was seen in glycylglycine buffer but not in phosphate and required high concentrations of both effectors, 30 mM DTT and 3 mM Fe.

This activation was not an in vitro curiosity. In the cell, L-SD was also synthesized in an inactive form. Two mutants previously described as being functionally defective in L-SD (23) were shown here to produce L-SD in a form which could be activated in vitro. It seems that these strains must be deficient in an activating system for which Fe and DTT can substitute.

The time course of L-SD activation was quite slow and was dependent on the concentration of Fe and DTT used,

over 30 min at the lowest concentration used and about 10 min with three times more of both Fe and DTT. Fe alone stimulated the reaction to some extent. DTT alone did not and indeed was inhibitory. The combination of the two gave the greatest activation.

Slow activation of enzymes on incubation is well known (1, 7, 26). The kinetics of activation of the α-ketoglutarate-dependent dioxygenases by Fe and DTT were very similar to the activation described here (1). Turner et al. described in detail the kinetics of activation of an enzyme of cephalosporin biosynthesis by preincubation with Fe, DTT, and ascorbate (28). A ring expandase involved in cephalosporin biosynthesis was similarly activated (16). However, these dioxygenases required α-ketoglutarate. Dialyzed inactive L-SD did not require α-ketoglutarate or any other cofactor (other than iron and DTT) to restore activity, so that L-SD is not likely to be one of this class of enzymes.

Many other enzymes have been shown to be activated in vitro by DTT and Fe, including δ-aminolevulinate dehydratase from radish (5), a sulfotransferase from *Rhodopseudomonas sulfidophila* (14), a chloroplast fructose 1,6-bisphosphatase (11), and a rat liver biliverdin reductase (8). In many of these cases, a naturally occurring electron carrier, thioredoxin, could replace DTT. The activation of beef heart aconitase has been shown to involve the reduction of 3Fe-4S clusters to 4Fe-4S clusters, which could be done with Fe, ascorbate, and a thiol (15).

Since the combination of Fe and DTT is a strong reducing agent, one would expect that activation involves reduction. Ribonucleotide reductase (RNR) could be reduced and activated both in vivo and in vitro by incubation with thioredoxin, and in vitro with DTT (12, 27). The reaction with thioredoxin forms part of the catalytic cycle of RNR. Electrons from NADPH are transferred to thioredoxin via the enzyme thioredoxin reductase. Reduced thioredoxin then interacts with RNR to produce the reduced, active form of the enzyme, which donates electrons to a ribonucleotide, reducing it to the corresponding deoxynucleotide. The function of RNR thus requires its cyclic oxidation and reduction.

By analogy, one would suggest that *E. coli* must normally make L-SD protein in an oxidized form, which would be reduced either by the physiological activating system in vivo or by an artificial substitute (Fe and DTT) in vitro. Strains MEW191 and MEW128 would then be able to synthesize the L-SD molecule but be unable to activate it.

Unlike RNR, however, the L-SD reaction does not involve reduction. Nothing is known about the mechanism of L-SD action, but it does not bring about a change in oxidation state, and a reducible disulfide would not seem essential. However, the cell may not be able to synthesize a reduced protein catalyzing L-SD directly and may have to produce the oxidized structure first, for some unknown structural reason.

Alternatively, the cell may have some enzymes which are only required to function at low redox potentials. It might build these in such a way that they require reduction to be activated. However, it is not clear why L-SD activity should be useful at low redox potential. In contrast, the main function of L-SD in the cell is not known. Although L-SD is a deaminase, it is induced by a variety of environmental challenges which may affect the redox state of the cell (20). L-SD induction might even be part of the oxidation stress syndrome (17).

The activating system for L-SD, whether structural or regulatory, has not yet been identified but might involve thioredoxin. Except that it causes resistance to T7 and to a filamentous phage (25), the mutational loss of thioredoxin and thioredoxin reductase had very little effect on the cell (3, 9, 18, 25). The mutations in strains MEW191 and 128 had very little effect on their phenotype (23) but did result in T7 resistance, allowing a very tentative suggestion that these mutations might affect thioredoxin.

The mixture of Fe and DTT is certainly a strong reductant. However, it may be a very complex mixture, as is "activated iron" produced by mixing an iron salt and hydrogen peroxide (Fenton's reagent) (29). The high concentration of Fe and DTT, and the fact that they must both be present together with the extract containing L-SD, might suggest that activation is due to a transitory species, present at low concentration in the Fe-DTT mixture.

ACKNOWLEDGMENTS

This work was supported by grant A6050 from the Canadian National Science and Engineering Research Council, for which we are very grateful.

We appreciate many years of discussion with P. Feigelson on the subject of L-SD. E.B.N. thanks the Marine Biological Laboratory of Woods Hole, Mass., for the use of its library and view.

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