Regulation of Isocitrate Dehydrogenase by Phosphorylation in *Escherichia coli* K-12 and a Simple Method for Determining the Amount of Inactive Phosphoenzyme

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In several *Escherichia coli* K-12 strains grown on a limiting concentration of glucose, isocitrate dehydrogenase (IDH) was inactivated about 90% after cessation of growth upon exhaustion of the glucose. Such inactivation has been previously observed in several *E. coli* strains but not in *E. coli* K-12 (unless acetate was added to the bacterial culture when growth ceased). IDH was inactivated 75 to 80% in all *E. coli* K-12 strains we examined during growth on acetate. The inactivation involved phosphorylation of the enzyme and is considered to be a regulatory mechanism facilitating metabolite flow along the glyoxylate shunt. Phospho-IDH interacted with antibodies to enzymatically active IDH. We have devised a method, based on this immunological cross-reaction, for determining the proportions of active and inactive (phospho-) IDH in cell extracts.

Isocitrate dehydrogenase (IDH; *threo*-D_s-isocitrate: NADP$^+$ oxidoreductase [decarboxylating]; EC 1.1.1.42) is a key enzyme of the tricarboxylic acid cycle because in a number of bacteria its substrate isocitrate can, in certain metabolic situations, be channeled along this cycle or via the glyoxylate shunt, which features the enzymes isocitrate lyase (ICL) and malate synthase (MS) (12). Hence, in such situations there will be competition between IDH and ICL for the common substrate, isocitrate, and regulation of the metabolite flow along the tricarboxylic acid cycle and the glyoxylate shunt becomes important. Evidence for such regulation came initially from the following observations. (i) When *Escherichia coli* ML308 was grown on limiting glucose, the IDH level remained high during growth; when growth ceased upon exhaustion of the glucose, however, ICL and MS activities were induced in cells metabolizing the acetate produced from glucose, and the IDH was largely inactivated. (ii) IDH activity was considerably lower in cells grown on acetate (which synthesized ICL and MS) than in glucose-grown cells, in which ICL and MS were repressed, and the lower activity was due to an inactivation of IDH (2, 10). Curiously, such IDH inactivation after cessation of growth on limiting glucose was seen in several enteric bacteria, including various strains of *E. coli* but not *E. coli* K-12 (2, 11). However, inactivation of IDH was observed in *E. coli* K-12 when acetate was added to the bacterial culture after growth ceased (7, 8). The inactivation of IDH resulted from its phosphorylation (16). We report here on IDH inactivation in several strains of *E. coli* K-12 grown on limiting glucose (without the addition of exogenous acetate) and on acetate and show that the inactivation is accompanied by phosphorylation of the enzyme. We have also devised an immunological method for determining the proportions of active and inactive IDH in cell extracts of the bacterium.

MATERIALS AND METHODS

Bacterial strains and cell growth. The following *E. coli* K-12 strains (with the genetic markers given in parentheses)

were used: wild type (F$^+$), W3899 (*nadB7* F$^-$), AT2473 (*thi-1 relA1 car-53* λ$^-$ *spoT1* Hfr), and C600 (*thr-1 leuB6 thi-1 lacY1* λ$^-$ F$^-$).

The bacteria were grown in salts medium (19) (the level of sodium citrate was 0.4 g/liter), supplemented as required for growth and containing the desired carbon source, with shaking at 37°C. When precise temperature control and timing of growth were necessary, a gyratory water bath shaker (New Brunswick Scientific Co., Inc., Edison, N.J.) was used. In experiments designed to label IDH with [^{32}P]phosphate, the growth medium was low in phosphate, containing 0.5 mM K_2HPO_4, and was buffered with 50 mM Tris hydrochloride at pH 7.5.

Preparation of cell extracts. In experiments in which the bacteria were grown on a limiting concentration of glucose, samples (1 ml) of the culture were withdrawn at the required times before and after cessation of growth, and each was added to a tube containing 1 ml of frozen buffer (5 mM potassium phosphate (pH 7.5) containing 5 mM $MgCl_2$ and 0.05% [wt/vol] sodium azide). This mixture was sonicated (Soniprep 150; MSE Instruments) for 1 min (the melting ice provided effective cooling) and used for enzyme and protein assays. By this procedure, the time between sampling and enzyme assay was reduced to a minimum of about 2 min; this was important for the IDH assays in view of the reported changes (11) in the activity of this enzyme in cell extracts. Larger volumes (8 ml) of culture samples were withdrawn when the cell extracts were also used for immunotitrations. These samples were cooled and centrifuged, and cell extracts were prepared as described below.

In experiments in which IDH levels were determined in cells growing on nonlimiting glucose or acetate or in which cultures were grown with $^{32}P_i$, the cultures were cooled and centrifuged. The sedimented cells were suspended in 10 mM potassium phosphate buffer (pH 7.5) containing 25% (wt/vol) sucrose and 5 mM EDTA, and cell extracts were prepared essentially as described previously (18).

Preparation of anti-*E. coli* IDH IgG linked to Sepharose. Antibodies were raised in rabbits to IDH purified (J. D. Edlin, Ph.D. thesis, University of Manchester, Manchester, United Kingdom, 1986) from a histidine-requiring auxotroph

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(6) of strain W3899, and the immunoglobulin G (IgG) was purified essentially as described earlier (20). The IgG was attached to Sepharose 4B by reaction with the cyanogen bromide-activated derivative of the Sepharose (Pharmacia, Uppsala, Sweden) (17).

Growth of strain W3899 in medium containing $^{32}P_i$ and isolation of IDH protein. An overnight culture of W3899 in low-phosphate medium containing 0.4% (wt/vol) glucose or sodium acetate as the carbon source was inoculated into 50-ml lots of low-phosphate medium containing as the carbon source either glucose at a limiting (0.05%) or nonlimiting (0.4%) concentration or sodium acetate at a nonlimiting (0.4%) concentration to give an A_{680} of 0.05. These fresh cultures were incubated at 37°C with shaking. $^{32}P_i$ (200 μCi) (Amersham International plc, Aylesbury, United Kingdom) was added to each culture containing limiting glucose at an A_{680} of 0.25 and to the other cultures at an A_{680} of 0.3, and incubation was continued. Growth stopped in the cultures containing limiting glucose at an A_{680} of about 0.5; cells were harvested by centrifugation at this point or 1 h later (IDH was inactivated during this postgrowth period). Cells from the other cultures were harvested at an A_{680} of 0.6 (i.e., in the exponential phase). From each lot of harvested cells, a cell extract (about 1 ml) was prepared.

A sample (0.5 ml) of the cell extract was allowed to enter a 1-ml column of anti-IDH IgG-Sepharose equilibrated with NET buffer (150 mM NaCl, 5 mM EDTA, 50 mM Tris hydrochloride [pH 7.5], 0.02% [wt/vol] sodium azide, 0.05% [vol/vol] Triton X-100) containing 5 mM $MgCl_2$, and the flow was then stopped for 2 h to facilitate binding of IDH to the column. The column was washed with NET buffer (20 ml) and the same buffer with no NaCl (20 ml). The column was then eluted with 4 M urea in 50 mM Tris hydrochloride (pH 7.5); 1 ml of the eluate was collected, and 0.5 ml was used for determination of radioactivity with a scintillation counter.

The eluates corresponding to the cultures grown on nonlimiting concentrations of glucose or acetate were also subjected to electrophoresis, after denaturation of the protein, in polyacrylamide gel containing sodium dodecyl sulfate. Protein in the gel was visualized by Coomassie brilliant blue, and radioactivity was visualized by autoradiography.

Immunotitration of cell extracts. The cell extract, diluted as necessary, was mixed with anti-*E. coli* IDH IgG, $MgCl_2$ (5 mM), and NET buffer in a final volume of 0.25 ml and incubated at 37°C for 15 min. Protein A adsorbent (0.25 ml) was then added, and incubation was continued for 15 min. The mixture was centrifuged at 10,000 × *g* for 2 min to sediment the protein A adsorbent and the IgG bound to it. The supernatant fraction was assayed for IDH activity. The protein A adsorbent was prepared as follows: Formalin-fixed *Staphylococcus aureus* cells (Bethesda Research Laboratories, Inc., Gaithersburg, Md.) were washed twice with NET buffer and resuspended in this buffer to a concentration of 1% (wt/vol).

Enzyme and protein assays. The reaction mixture for IDH assay contained 20 mM potassium phosphate buffer (pH 8), 10 mM $MgCl_2$, 0.17 mM $NADP^+$ (sodium salt), enzyme sample, and 2 mM trisodium DL-isocitrate in a total volume of 1 ml at 30°C. One unit of IDH produced 1 μmol of NADPH per min under these assay conditions. ICL was assayed at 30°C by a continuous spectrophotometric method (1 U of enzyme produced 1 μmol of glyoxylate per min) (5), and MS was assayed by a method in which the glyoxylate-dependent cleavage of acetyl coenzyme A to coenzyme A was followed spectrophotometrically at 232 nm (1 U of enzyme produced 1 μmol of coenzyme A per min) (4).

Protein was determined by the biuret method (9) or by the Coomassie blue binding assay (3). Specific enzyme activity was expressed as units per milligram of protein.

RESULTS

IDH inactivation in *E. coli* K-12 strains after cessation of growth on limiting glucose. The specific IDH, ICL, and MS activities in extracts of cells of strain W3899 sampled during growth and after cessation of growth due to exhaustion of the limiting concentration of glucose are shown in Fig. 1. In less than 1 h after growth stopped, IDH in the cells was inactivated more than 90%; during this period, the glyoxylate shunt enzymes, ICL and MS, were synthesized to an appreciable level. IDH activity remained at the lowest level for about 1 h and thereafter recovered gradually toward the high level seen in cells growing on glucose. The level of malate dehydrogenase (another tricarboxylic acid cycle enzyme), however, remained constant during and after cessation of growth on limiting glucose. Similar inactivation of IDH occurred in the wild-type strain and strain AT2473. By contrast, specific IDH activity in cell extracts of strain C600 remained virtually constant at about 0.8 U/mg of protein during growth on glucose and for at least 2.5 h after cessation of growth.

IDH inactivation in the three K-12 strains was similar to that found in *E. coli* ML308. The analogy between the two systems is further heightened by our observation that when L-malate, a carbon source readily utilized by *E. coli*, or chloramphenicol, which inhibits protein synthesis, was added to a culture of strain W3899 on limiting glucose upon cessation of growth, inactivation of IDH was strongly arrested, as found in similar experiments with *E. coli* ML308 (10). The interpretation given for ML308, i.e., that a significant proportion of the glucose carbon accumulates as acetate in the culture and that after cessation of growth the acetate is utilized, with the induction of ICL and MS and the inactivation of IDH (10), may therefore be assumed to be valid for *E. coli* K-12 as well. Moreover, as in the ML308 system (16), inactivation of IDH in *E. coli* K-12 involved phosphorylation of the enzyme (see below).

IDH levels in *E. coli* K-12 strains grown on glucose and on acetate. The specific activities of IDH, ICL, and MS in extracts of cells of several *E. coli* K-12 strains that were actively growing on glucose or on acetate are presented in Table 1. In all strains examined, IDH activity during growth on acetate was less than 25% of the activity during growth on glucose, and, as expected, ICL and MS were induced only in cells growing on acetate. Addition of glucose or pyruvate to a culture of W3899 growing on acetate led to a prompt rise in IDH activity (data not shown). Similar results were observed in the ML308 system (2). When the added glucose (1 mM) or pyruvate (2 mM) was consumed, IDH activity dropped toward the low level in acetate-grown cells. The possibility that the low IDH activity in acetate-grown cells was due to inactivation of the enzyme by phosphorylation was investigated.

IDH phosphorylation in acetate-grown cells and in cells grown on limiting glucose after cessation of growth. Strain W3899 was grown in medium containing glucose (limiting or nonlimiting) or nonlimiting acetate, with addition of $^{32}P_i$. IDH protein was isolated from cells by using an anti-IDH IgG column, and the radioactivity in the protein was determined (see Materials and Methods). $^{32}P_i$ was incorporated into IDH protein during growth on acetate (8,604 cpm of radioactivity in 1 ml of urea-Tris hydrochloride eluate from
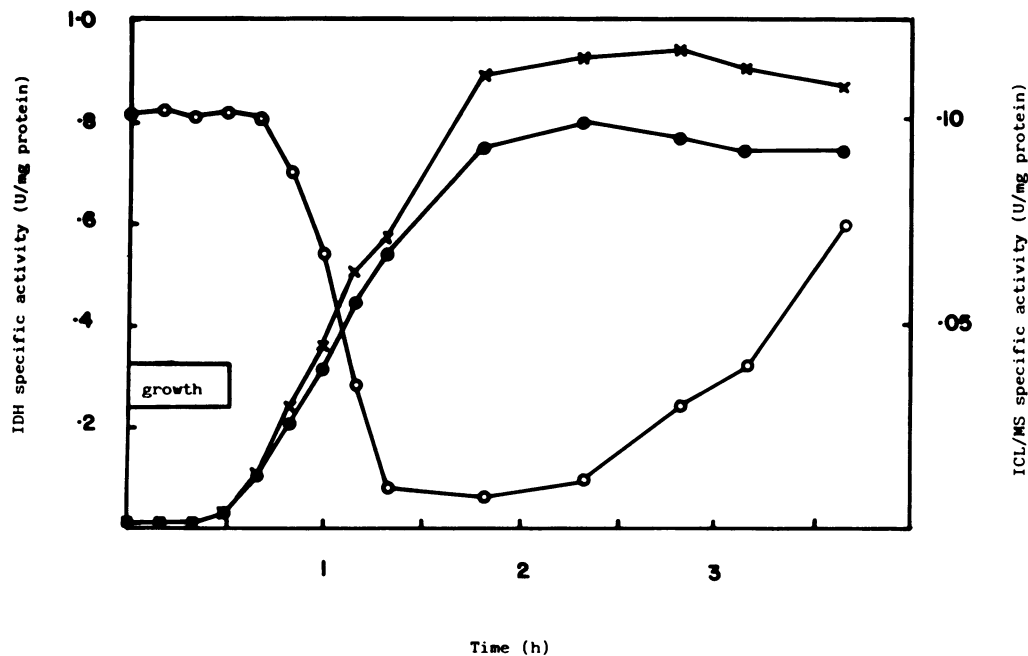
Time (h)

FIG. 1. IDH (○), ICL (●), and MS (×) levels in cells of *E. coli* K-12 W3899 during growth and after cessation of growth on limiting glucose. Salts medium containing a limiting (0.05%) concentration of glucose was inoculated from an overnight culture to an A_{680} of 0.02 to 0.05 and incubated with shaking at 37°C. A_{680} at time zero was 0.35, and growth stopped at an A_{680} of about 0.5. Cell extracts prepared from culture samples withdrawn at various times were assayed for IDH, ICL, and MS and for protein by the Coomassie blue binding method.

the anti-IDH IgG column) and while IDH was inactivated (for 60 min) after cessation of growth on limiting glucose (10,568 cpm of radioactivity) but not during growth on glucose (112 cpm of radioactivity). The following observations confirm that the protein isolated from the anti-IDH IgG column was indeed IDH protein. When a column of preimmune IgG instead of the anti-IDH IgG column was used for fractionation, the eluate from the extract of cells grown on acetate with $^{32}P_i$ had virtually no radioactivity (84 cpm). When the eluates from the anti-IDH IgG column corresponding to the extracts of cells grown on nonlimiting glucose and on acetate were analyzed by electrophoresis, after denaturation of the protein, in polyacrylamide gels containing sodium dodecyl sulfate, a single protein band was visualized by Coomassie blue in each case. The position of each protein band corresponded to that of the band yielded by an authentic sample of pure *E. coli* IDH. However, only the band from the acetate-grown cells was radioactive (Fig. 2). These

findings lead to the conclusion that in *E. coli* K-12, as in *E. coli* ML308 (16), IDH inactivation was the concomitant of its phosphorylation and that in acetate-grown cells of W3899 (and presumably of the other *E. coli* K-12 strains listed in Table 1), IDH was inactivated to a substantial extent.

Quantitative determination of active and inactive IDH in cell extracts of *E. coli* K-12 strains by immunotitration. Figure 3 presents plots obtained from immunotitration of cell extracts prepared from culture samples taken at 30 and 110 min in the experiment shown in Fig. 1. The appreciably less steep plot for the 110-min sample, in which more than 90% of the IDH was in the inactive form, suggests that the inactive phospho-IDH interacted with the anti-IDH IgG. In this experiment, since the two samples used in the immunotitrations were adjusted to have the same enzyme activity per milliliter, the protein concentration in the 110-min sample was some 12-fold higher than that in the 30-min sample. The possibility that the difference in slope between the plots might have

TABLE 1. Active IDH levels and active IDH as a fraction of total IDH protein in *E. coli* K-12 strains grown on glucose and on acetate[a]

E. coli strain	Specific enzyme activity (U/mg of protein)						Proportion of active IDH (%)	
	IDH		ICL		MS			
	Glucose	Acetate	Glucose	Acetate	Glucose	Acetate	Glucose	Acetate
W3899	0.82	0.19	0.007	0.22	ND[b]	0.28	100	22
AT2473	0.81	0.17	0.006	0.23	ND	0.29	98	20
Wild type	0.83	0.17	0.004	0.23	ND	0.30	100	20
C600	0.83	—[c]	—	—	—	—	98	24

[a] Cultures were grown in salts medium containing glucose or acetate (both at nonlimiting concentrations) as the carbon source, using overnight cultures as inocula, through about four generations to midexponential phase. Cell extracts were assayed for IDH, ICL, and MS and for protein by the biuret method. The extracts were also adjusted, by suitable dilution, to contain the same IDH activity per milliliter and used in immunotitrations against anti-*E. coli* IDH IgG for determining the proportions of active IDH.

[b] ND, Not detected.
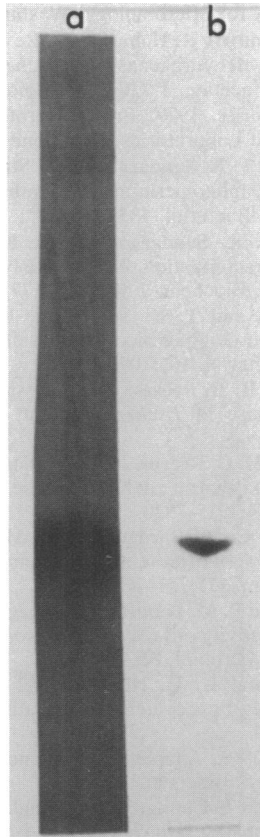
[c] —, Not determined.

FIG. 2. Electrophoresis and autoradiography of eluates from anti-*E.coli* IDH IgG column of extracts of cells of *E. coli* K-12 W3899 grown with $^{32}P_i$ and glucose (0.4%) or sodium acetate (0.4%). The eluates were subjected to electrophoresis, after denaturation of the protein, in polyacrylamide gels containing sodium dodecyl sulfate, and protein and radioactivity in the gels were visualized (see Materials and Methods). The bands of radioactivity (a) and protein (b) in the gel corresponding to the acetate culture are shown. In the gel corresponding to the glucose culture (not shown), there was a similar protein band but no band of radioactivity.
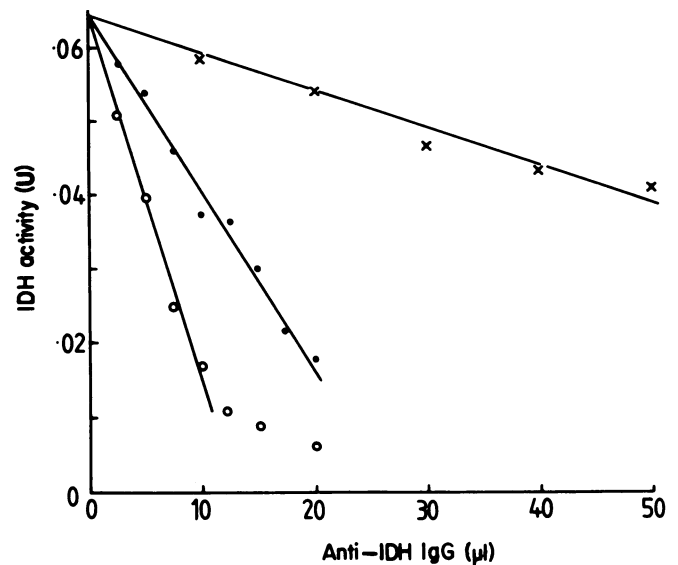

FIG. 3. Immunotitration of extracts of cells of *E. coli* K-12 W3899 from a culture on limiting glucose. Cell extracts prepared from culture samples withdrawn at 30 and 110 min in the experiment described in the legend to Fig. 1 were adjusted by suitable dilution to have the same IDH activity per milliliter and used for immunotitration against anti-*E. coli* IDH IgG. In the immunotitration, the antibody-IDH complex was sedimented with a protein A preparation, and the IDH activity remaining in the supernatant fraction was determined. The IgG preparation contained 0.2 mg of protein per ml. Symbols: O, cell extract from 30-min culture sample; ×, cell extract from 110-min culture sample; ●, 1:1 mixture of the two extracts.

resulted from this large disparity in protein concentration rather than from a specific interaction of inactive IDH with the antibodies was ruled out by the following observations. A 1:1 mixture of the two samples yielded a plot with more or less the slope expected from a specific interaction (Fig. 3). Also, when the immunotitrations were performed with the two samples at the same protein concentration, plots differing in slope to an extent similar to that in Fig. 3 were obtained. Moreover, the inclusion of bovine serum albumin at a concentration of 5 mg/ml did not alter the slope. The inability of IgG prepared from preimmune serum to inhibit IDH activity suggests that the observed inhibition by anti-IDH IgG was specific.

Results of the IDH activity assays indicated that the specific activity of the 110-min sample was about 7% that of the 30-min sample, which contained all of the IDH in the active form. The proportions of active and inactive IDH in the former sample were therefore about 7 and 93%, respectively. Assuming that the slope of the immunotitration plot for the 30-min sample corresponded to 100% active IDH, then the slope of the plot for the 110-min sample was equivalent to an active IDH content of 8% (and an inactive IDH content of 92%), which is in good agreement with the result from IDH activity assays. For another sample taken at

60 min (see Fig. 1), the proportions of active and inactive IDH were 65 and 35% as determined by activity assay and 64 and 36% as estimated from the slope of the immunotitration plot (not shown); again, the two methods of analysis yielded essentially the same result. This immunotitration method was therefore used for determining the proportions of active and inactive IDH in bacterial cell extracts.

Table 1 shows the fractions of the IDH protein present as the enzymatically active form in *E. coli* K-12 cell extracts made from cultures grown with nonlimiting concentrations of glucose or of acetate, as determined by the immunotitration method. In all cases in which the carbon source was glucose, all of the IDH was in the active form. By contrast, when the bacteria were grown on acetate, the proportion of active IDH was only 20 to 25%. This result provides further support for the conclusion that the low IDH activity observed in acetate-grown cells (Table 1) was due to inactivation of the enzyme by phosphorylation. The proportions of active IDH in cells of the wild type and strain AT2473 grown on limiting glucose were also determined by immunotitration at different stages of inactivation after exhaustion of the glucose. The results (data not shown) were similar to those obtained with strain W3899.

DISCUSSION

This investigation establishes that *E. coli* K-12 is similar to *E. coli* ML308 and certain other enteric bacteria in inactivating IDH upon cessation of growth on limiting glucose (without the addition of exogenous acetate) and that this inactivation is due to phosphorylation of the enzyme. IDH inactivation by phosphorylation has been observed previously in *E. coli* K-12, but after addition of acetate to a culture

grown to stationary phase on limiting glucose under conditions in which there was no net cell growth (8). The failure of previous workers to observe IDH inactivation in *E. coli* K-12 upon exhaustion of limiting glucose without addition of acetate was possibly because in their experiments the *E. coli* derivatives used did not accumulate acetate while utilizing the glucose. Our failure to see IDH inactivation in strain C600 may have the same explanation. Apparently, some *E. coli* K-12 strains behave differently. The three strains (wild type, W3899, and AT2473) that inactivated IDH upon exhaustion of glucose in our study represent the three sex types F$^+$, F$^-$, and Hfr. Therefore, the discrepant behaviors of strains like C600 are unlikely to be due to sex type. Strain variations in *E. coli* K-12, resulting in significant differences in characteristics, are perhaps not totally surprising in view of the complicated and varied history of the use of this bacterium (involving the derivation of numerous lines) in research over several decades (1). IDH inactivation by phosphorylation, however, occurs in all *E. coli* K-12 strains examined by us, including strain C600 (Table 1), when these strains are grown on acetate. LaPorte et al. (15) have demonstrated the presence of phospho-IDH in acetate-grown *E. coli* K-12 on the basis of an increase in the IDH activity of extracts of acetate-grown cells upon incubation with IDH kinase-phosphatase. The immunotitration method devised by us offers a good alternative means for determining the relative amounts of the active and inactive forms of IDH.

It is evident from the levels of active IDH and the proportions of active IDH presented in Table 1 that for the wild type and strains W3899 and AT2473 (and presumably for other *E. coli* K-12 strains), the level of IDH protein (active and inactive) is about the same in cells grown on glucose and on acetate. When the cells are grown on acetate, 75 to 80% of the IDH is inactivated by phosphorylation. Such regulation of IDH activity is rationalized teleologically in terms of facilitating the metabolite flow along the glyoxylate shunt. Mechanistically, it can be explained by the recent findings that the gene coding for the single enzyme that both phosphorylates IDH and dephosphorylates phospho-IDH is part of the operon that carries the genes for the glyoxylate shunt enzymes, ICL and MS, which are induced by acetate, and that the phosphorylating and dephosphorylating activities are affected in opposite ways by metabolites such as isocitrate and 3-phosphoglycerate (13–16).

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