The pseudowords were matched to the real words in probability of occurrence of each individual letter. The letter strings consisted of consonants. The false-font characters were composed of contiguous curved and straight-line segments designed to be at once dissimilar from any of the alphabetical symbols, but equivalent in the content of primitive visual features. Each character was generated on a 7 by 5 pixel grid. All four stimulus sets were matched in average number of pixels illuminated; vertical, horizontal, and oblique contours; and approximately matched in curvature, corners, and line segment intersections. These stimuli were presented once per second with an on-time of 150 ms.

14. The area of left, medial temporal cortex activated by visually presented words and pseudowords was composed of several identifiable foci of blood flow change. The stereotactic atlas coordinates of these foci were based on our system of anatomical localization with PET. The coordinates (z, x, y) were for real words: (+2, +29, −53), (+2, +21, −63), and (+6, +21, −41) for pseudowords: (+4, +23, −65) and (−47, +4, +19).
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Electrostatic and Steric Contributions to Regulation at the Active Site of Isocitrate Dehydrogenase

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The isocitrate dehydrogenase of *Escherichia coli* is regulated by covalent modification at the active site rather than, as expected, at an allosteric site. As a means of evaluating the mechanism of regulation, the kinetics of the substrate, 2*R*,3*S*-isocitrate, and a substrate analog, 2*R*-malate, were compared for the native, phosphorylated, and mutant enzymes. Phosphorylation decreases activity by more than a factor of 10^6 for the true substrate, but causes minor changes in the activity of the substrate analog. The kinetic results indicate that electrostatic repulsion and steric hindrance between the phosphoryl moiety and the γ carboxyl group of 2*R*,3*S*-isocitrate are the major causes of the inactivation, with a lesser contribution from the loss of a hydrogen bond.

COVALENT REGULATION, PARTICULARLY by phosphorylation, is ubiquitous in biological systems (*1*). The discovery of covalent modification at the active site (*2*) raises questions concerning the mechanism of regulation. Phosphorylation of a Ser^{113} completely inactivates the isocitrate dehydrogenase (IDH) of *Escherichia coli* (*3–5*) by preventing isocitrate binding (*6*) and without significant conformational changes (*7*). Substitution for Ser^{113} by aspartate and glutamate also inactivates IDH, whereas substitutions by other amino acids do not (*5, 6*). It was postulated that electrostatic factors may play a dominant role in inactivation (*5*), and calculations suggest that such a repulsion could explain the findings (*2*).

Given the uncertainties in the electrostatic calculations on complex surfaces (*8*), independent corroborative evidence is required for assessing the relative contributions of various factors. To do this, the kinetic properties of a substrate, 2*R*,3*S*-isocitrate, and a substrate analog, 2*R*-malate, were compared with the use of native, phosphorylated, and mutant enzymes. Since catalysis occurs at the α and β carboxyl groups of 2*R*,3*S*-isocitrate, a compound (2*R*-malate) retaining the reactive groups of 2*R*,3*S*-isocitrate, but lacking the γ carboxyl that interacts with the phosphoryl moiety, might bind and serve as a substrate for the phosphorylated enzyme (Fig. 1).

Indeed 2*R*-malate, which has the same chirality as 2*R*,3*S*-isocitrate, is a substrate for IDH, whereas 2*S*-malate is not. As expected, the Michaelis constant (K_m) of the substrate analog is larger, and the maximum velocity is smaller than for the natural substrate isocitrate (Table 1). However, 2*R*-malate is also a substrate for the phosphorylated enzyme, whereas 2*R*,3*S*-isocitrate is not. In fact, phosphorylation has little effect on the Michaelis constant for 2*R*-malate (Table 1). Whereas substitutions with different amino acids for Ser^{113} have dramatic effects on the kinetic constants for 2*R*,3*S*-isocitrate, the effects on the kinetic constants for 2*R*-malate are much smaller. Thus, direct interactions between the γ carboxyl group of isocitrate and the side chain of residue 113 are primarily responsible for the regulation of IDH activity (Fig. 1).

The effects of phosphorylation can now be addressed quantitatively. Approximately, twofold changes in the isocitrate Michaelis constant and maximum activity can be attributed to the loss of the hydrogen bond with the γ carboxyl group of isocitrate as determined by the replacement of Ser^{113} with alanine ($V_{max}/V_{max\text{-}Ser} = 0.08$; $K_{m\text{-}Ala}/K_{m\text{-}Ser} = 1.8$ (*5*). The crystallographic structures show that overlaps between the Van der Waals radii of the side chains of glutamate, aspartate, phosphoserine, tyrosine, and lysine at site 113, and the γ carboxyl group of isocitrate are possible (*2*). The tyrosine and lysine residues, which have large overlaps, cause decreases in V/K_m by factors of 8.5×10^3 and 3.5×10^3, respectively (Table 1). The positively charged amino group of lysine may have an activating effect, but this is probably small because the amino group is so far removed from the γ

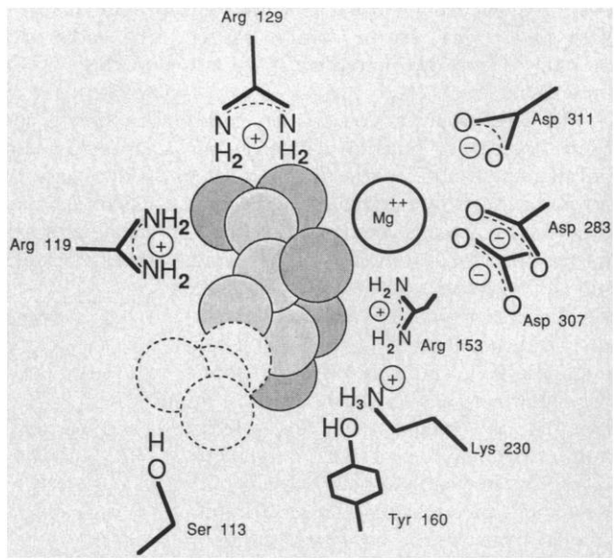

Fig. 1. 2*R*-malate (shaded spheres) and 2*R*,3*S*-isocitrate (shaded and dashed spheres) in the active site of IDH. The γ carboxyl group of 2*R*,3*S*-isocitrate (dashed spheres) hydrogen bonds to Ser^{113}, which is also the site of phosphorylation. The α, β, and γ carboxyl groups of 2*R*,3*S*-isocitrate hydrogen bond to three Arg^{119}, Arg^{129}, and Arg^{153}. The α carboxyl group and α hydroxyl group are chelated to a magnesium ion associated with Asp^{283} and Asp^{307} and the β carboxyl group hydrogen bonds to Tyr^{160} and Lys^{230}. During catalysis, the loss of a proton from the α hydroxyl group and the transfer of the hydrogen from the α carbon to nicotinamide adenine dinucleotide phosphate (NADP) results in dehydrogenation. The loss of the β carboxyl group as CO_2 is followed by protonation of the β carbon to form α-ketoglutarate.

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carboxyl group of 2*R*,3*S*-isocitrate.

Replacements with aspartate and glutamate, which are smaller but carry single negative charges, cause changes in V/K_m by factors of 7×10^5 and 2×10^5, respectively (Table 1). The glutamate side chain is one CH_2 group longer than that of aspartate, but yields a slightly more active enzyme. Presumably, the extra CH_2 group of the glutamate increases the distance between the two repulsive groups. This suggests that steric hindrance is less important than the electrostatic effects for these substitutions.

Because the phosphoserine is as bulky as tyrosine and carries two negative charges, a factor of 10^4 for steric hindrance and a factor of 10^5 for electrostatic effects may give a net factor of $10^4 \times 10^5 = 10^9$. This is consistent with the complete inactivation of the enzyme that is observed. Thus, both steric and electrostatic effects make important contributions to inactivation by phosphorylation.

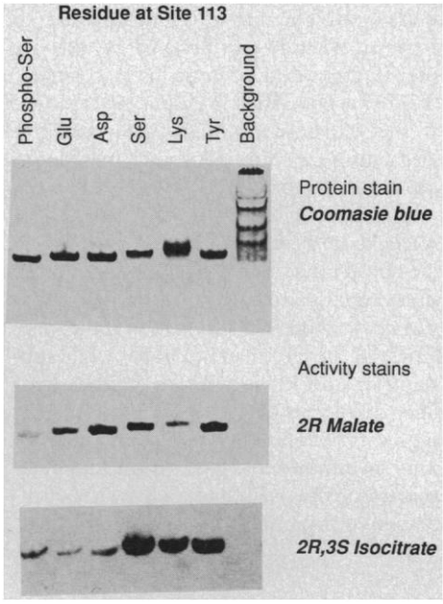

Fig. 2. The demonstration that 2*R*-malate is an alternate substrate for IDH by means of native polyacrylamide gel electrophoresis (*11*) of purified mutant enzymes. The gel was 7.5 percent acrylamide with 2.5 percent bis and 0.1 M tris base with boric acid to pH 7.8. The running buffer was 0.05 M tris base with boric acid to pH 7.8. Approximately 0.2 mg of protein in 5 μl of 10 percent glycerol with 0.002 percent bromophenol blue were placed on each lane and subjected to electrophoresis at 20 V/cm at about 0.03 A for 45 min at 4°C. Coomassie blue was used as a protein stain (25 percent isopropanol, 10 percent acetic acid, 0.05 percent Coomassie blue for 1 hour and destained with 10 percent acetic acid). The gels were washed in tris-HCl, pH 7.6, for 1 hour, and then stained for enzyme activity (0.1 M tris-HCl, pH 7.6, with 0.25 mM nitrotetrazolium blue and 0.078 mM phenazine methosulfate, 1 mM NADP, and either 5 mM $MgCl_2$ and 1 mM 2*R*,3*S*-isocitrate or 20 mM $MgCl_2$ and 50 mM 2*R*-malate (*12*), until blue bands appeared as a result of the precipitation of nitrotetrazolium blue and phenazine methosulfate when reduced by the NADPH from active dehydrogenase reactions.

Native polyacrylamide gel electrophoresis of the mutant enzymes provides a test to eliminate the possibility that 2*R*-malate is a substrate for a contaminating dehydrogenase. The mobilities of the mutant enzymes are altered when the neutral Ser^{113} is replaced by amino acid residues with charged side chains. Yet the bands on gels stained for 2*R*-malate dehydrogenase activity follow those of the protein stain precisely (Fig. 2). Any contaminating enzyme that happened to have the same mobility as the native wild-type enzyme would have failed this test.

Gels stained for IDH activity support these conclusions and reveal an interesting feature of phosphorylated enzyme. Phosphorylated IDH is prepared directly from the active native enzyme, which is a dimer (*4–6*). The mobilities of the bands stained for 2*R*-malate dehydrogenase activity and Coomassie blue protein stain coincide. However, when stained for IDH activity with 2*R*,3*S*-isocitrate, the band appears retarded and comigrates with those of the glutamate and aspartate mutants (carrying two extra negative charges per dimer, as opposed to four). This suggests that the IDH activity can be ascribed to a heterodimer with only one phosphorylated subunit. Moreover, the Michaelis constant toward isocitrate (Table 1, footnote) is similar to that of native enzyme. The absence of IDH activity stain at the position occupied by the fully phosphorylated dimer indicates it is completely inactive, as predicted from the kinetic results. The previously described activities (*5*) of the aspartate and glutamate mutant enzymes were compromised by the presence of small amounts of the wild-type enzyme. Here, the aspartate and glutamate mutant enzymes were harvested from a strain carrying a deletion eliminating all of the chromosomal IDH gene (strain JLK1). The residual IDH activities of these mutant enzymes were not caused by contamination because their relative electrophoretic mobilities, as indicated by staining for IDH activity, are identical with those of the protein stain (Fig. 2).

The kinetic evidence reported here confirms that phosphorylation of a serine immediately adjacent to the active site of IDH regulates activity by direct interactions with the substrate at the active site. A major factor is the electrostatic repulsion between the negatively charged phosphoryl group and the negatively charged γ carboxyl group of isocitrate. Steric hindrance between these groups also plays a role. Both factors may be important in other enzymes. For example, tyrosine kinase pp34, which plays a central role in the cell cycle, contains the Gly-X-Gly-X-X-Gly of a typical ATP binding domain and is inactivated by phosphorylation of a tyrosine at the third X site (*9*). Other kinases may be regulated by similar mechanisms. It

Table 1. Kinetic parameters of mutant enzymes toward 2*R*,3*S*-isocitrate and 2*R*-malate. Site-directed mutants were introduced into plasmid pTK513 (*4*) by the uridine-labeled template method (*13*) and screened by dideoxy sequencing (*14*). The enzymes were purified by the method of Garnak and Reeves (*15*) with minor modifications (*4, 5*). Initial rates were determined in 25 mM MOPS, 100 mM NaCl, 2mM DTT, 2.5 mM NADP, pH 7.3 at 25°C. The range in concentration of 2*R*,3*S*-isocitrate was from 2 μM to 200 mM in the presence of 5 mM $MgCl_2$, and the range for 2*R*-malate was from 3 mM to 100 mM in the presence of 50 mM $MgCl_2$. Each standard error is no more than 10 percent of its respective estimate.

Residue at site 113	Observed parameters				Parameters normalized to Ser^{113}			
	2*R*,3*S*-Isocitrate		2*R*-Malate		2*R*,3*S*-Isocitrate		2*R*-Malate	
	V_{max} (μM/min·mg)	K_m (μM)	V_{max} (μM/min·mg)	K_m (μM)	V_{max} / $V_{max\text{-}Ser}$	K_m / $K_{m\text{-}Ser}$	V_{max} / $V_{max\text{-}Ser}$	K_m / $K_{m\text{-}Ser}$
Ser	77600	5.2	7.3	6700	1	1	1	1
Phospho-Ser	*	*	0.1	11500			0.014	1.7
Glu	85	950	2.0	9700	0.0011	183	0.27	1.4
Asp	45	2300	11.0	16200	0.0006	444	1.5	2.4
Lys	2500	600	0.5	22200	0.0320	115	0.07	3.3
Tyr	730	420	2.7	48400	0.0094	81	0.37	7.2

*Direct measurement of the phosphorylated enzyme gave a V_{max} of 110 μM/min·mg and a K_m of 7.1 μM. This K_m value, unlike those of the ASP and Glu mutants, is close to that of the wild type and suggests that this residual activity is caused by approximately 0.1 percent dephosphorylated enzyme in the preparation.

is also possible that an enzyme could be activated when phosphorylated at an active site, if the substrate contains a positively charged group. Thus, covalent modification at the active site provides an alternative mechanism to the allosteric mechanism demonstrated for phosphorylase (*10*).

Site-directed mutagenesis provides a means to analyze the mechanisms by which the phosphorylation of serine residues regulates protein function. Substitution with aspartate or glutamate should reveal the magnitude of the electrostatic effects, while substitution with bulky residues, such as tyrosine, should analyze the steric effects. The approach becomes particularly powerful if a combination of substrate analogs and mutagenized protein is used to evaluate the quantitative contribution of each factor.

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Presence of a Potent Transcription Activating Sequence in the p53 Protein

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The p53 gene is frequently mutated in a wide variety of human cancers. However, the role of the wild-type p53 gene in growth control is not known. Hybrid proteins that contain the DNA binding domain of yeast GAL4 and portions of p53 have been used to show that the p53 protein contains a transcription-activating sequence that functions in both yeast and mammalian cells. The NH_2-terminal 73 residues of p53 activated transcription in mammalian cells as efficiently as the herpes virus protein VP16, which contains one of the strongest known activation domains. Combined with previous data that showed p53 is localized to the nucleus and can bind to DNA, these results support the idea that one function of p53 is to activate the transcription of genes that suppress cell proliferation.

THE CELLULAR PROTEIN P53 IS INvolved in normal cell growth control and in transformation. It was originally identified as a protein that forms a stable complex with the SV40 large tumor antigen and the adenovirus E1b protein (*1*). In many transformed cells, p53 is found with a much increased half-life and in correspondingly elevated concentrations (*2*). Although introduction of p53 into primary cells was shown to result in immortalization and (in cooperation with an activated *ras*) to result in transformation (*3*), experiments have shown that these p53 genes contained mutations (*4*). The wild-type p53 gene is incapable of transforming cells and can inhibit transformation by mutant p53 genes and other oncogenes (*5*). In addition, mutant versions of the p53 gene are found in a large variety of human tumors, and the encoded proteins may inactivate the wild-type p53 protein by forming inactive oligomeric complexes (*6*). Thus wild-type p53 is classified as a tumor suppressor gene or recessive oncogene, whose inactivation can lead to the transformation process (*5, 6*).

The biochemical mechanisms by which p53 acts in cell proliferation are as yet unknown. The protein is mainly localized to the nucleus and at least some fraction of the protein appears to be associated with chromatin or the nuclear matrix (*7*). The p53 protein is multiply phosphorylated (*8*) and is capable of binding to double-stranded and single-stranded DNA (*9*). In cotransfection assays with a reporter gene containing the long terminal repeat (LTR) promoter of an intracisternal A particle, p53 was able to increase the activity of the reporter gene (*10*). However, these experiments did not provide evidence that p53 specifically affected transcription, nor did they show whether the observed enhancement was a direct or indirect effect of the transfected p53. Although p53 may function as an activator of transcription, a direct test of this function cannot be performed in the absence of knowledge of its target genes. We now show that p53 has an activating domain by constructing hybrid proteins between portions of p53 and a heterologous DNA binding domain, in this case the DNA binding domain from the yeast protein GAL4.

For assaying transcriptional activation in yeast, we used strain GGY1::171 (*11*), which is deleted for *GAL4* and contains an integrated *GAL1-lacZ* under the regulation of the GAL4 protein (Fig. 1A). The source of p53 sequences was the plasmid pR4-2 (*12*), which contains a cDNA copy of a mutant p53 mRNA from the human A431 cell line. The mutation is His instead of Arg at residue 273, which has also been observed in breast and colon tumors (*6*). We constructed five plasmids (*13*) encoding GAL4-p53 hybrids that could be expressed in yeast (Fig. 1B), using as a parental vector the plasmid pMA424 (*14*). Vector pMA424 contains the yeast *ADH1* promoter fused to a fragment of the *GAL4* gene encoding the NH_2-terminal domain (amino acids 1 to 147) of GAL4. This region of GAL4 (designated $GAL4_D$) is capable of localizing to the nucleus and binding to specific sequences upstream of the *GAL1-lacZ* gene, but will not itself activate transcription (*15*). Thus β-galactosidase activity is a measure of the activating function of the sequences fused to the DNA binding domain. In addition, pMA424 carries sequences for selection in yeast and for replication in high copy. As controls, we used the GAL4 DNA binding domain alone and the entire GAL4 protein.

The wild-type GAL4 protein activated significant transcription of the *GAL1-lacZ* gene, and hybrids containing the first 137 amino acids, 346 amino acids, or all (393 amino acids) of the p53 protein activated to varying degrees, ranging from approximately

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