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Catalytic properties of an *Escherichia coli* formate dehydrogenase mutant in which sulfur replaces selenium

(selenocysteine/selenoenzymes/TGA codon/kinetics)

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ABSTRACT **Formate dehydrogenase H of *Escherichia coli* contains selenocysteine as an integral amino acid. We have purified a mutant form of the enzyme in which cysteine replaces selenocysteine. To elucidate the essential catalytic role of selenocysteine, kinetic and physical properties of the mutant enzyme were compared with those of wild type. The mutant and wild-type enzymes displayed similar pH dependencies with respect to activity and stability, although the mutant enzyme profiles were slightly shifted to more alkaline pH. Both enzymes were inactivated by reaction with iodoacetamide; however, addition of the substrate, formate, was necessary to render the enzymes susceptible to alkylation. Alkylation-induced inactivation was highly dependent on pH, with each enzyme displaying an alkylation vs. pH profile suggestive of an essential selenol or thiol. Both forms of the enzyme use a ping-pong bi-bi kinetic mechanism. The mutant enzyme binds formate with greater affinity than does the wild-type enzyme, as shown by reduced values of K_m and K_d. However, the mutant enzyme has a turnover number which is more than two orders of magnitude lower than that of the native selenium-containing enzyme. The lower turnover number results from a diminished reaction rate for the initial step of the overall reaction, as found in kinetic analyses that employed the alternative substrate deuterioformate. These results indicate that the selenium of formate dehydrogenase H is directly involved in formate oxidation. The observed differences in kinetic properties may help explain the evolutionary conservation of selenocysteine at the enzyme's active site.**

Selenium is a required micronutrient for mammals, birds, and bacteria (1–3). Selenium deficiencies cause white muscle disease and infertility in domestic mammals and cardiomyopathies in humans. Proper thyroid hormone function requires an adequate amount of selenium in the diet (4). Selenium is essential for the proper function of the immune system as well as for cellular defense against oxidative damage, and therefore this element may play a role in the prevention of cancer and premature aging.

Selenium deficiencies affect health because the synthesis and activity of certain enzymes require selenium. Most known selenium-dependent enzymes contain selenocysteine, a cysteine analog in which selenium replaces sulfur (5).

Selenocysteine incorporation into proteins is directed by a UGA codon, which is normally regarded as a stop codon (6, 7). However, this is not a case of termination suppression, because selenocysteine incorporation into polypeptides requires a specific tRNA capable of recognizing the UGA codon in unique contexts (8–10). Selenocysteine has been called the 21st amino acid essential for ribosome-directed protein synthesis (11).

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A wide variety of species have retained selenocysteine as an essential component of crucial proteins. This evolutionary conservation may be due to the fact that selenocysteine has unique chemical properties which are not shared by other amino acid functional groups. Selenium resides directly below sulfur in group VI-A of the periodic table. The two elements have many of the same valence states, they undergo many of the same reactions, and they form analogous organic derivatives that are isomorphic. However, organoselenium compounds are generally more reactive, particularly in oxidation–reduction reactions. With respect to acid dissociation constants, the selenol of free selenocysteine has a pK_a of 5.2, while the thiol of free cysteine has a pK_a greater than 8. Therefore, at physiological pH values a selenol group is almost fully ionized, while a thiol group is largely protonated.

Little is known about the properties of selenocysteine as a functional component of proteins. In subtilisin, chemical conversion of the active-site serine to selenocysteine modified the enzyme from a protease into an acyl transferase (12), which also exhibited peroxidase activity (13). Replacing the cysteines of a metallothionein with selenocysteines resulted in a protein that bound copper tightly, but fewer copper atoms were bound per molecule of the selenium protein compared with the native protein (14). These examples show that selenocysteine can have a profound effect on a protein's activity.

We have undertaken to define some of the unique biochemical properties of selenium, using formate dehydrogenase H ([Se]FDH_H) as a model selenoprotein. [Se]FDH_H is produced by *Escherichia coli* grown under anaerobic conditions in the absence of added electron acceptors (15). This selenoenzyme is a component of the formate hydrogen lyase complex, which decomposes formate to carbon dioxide and molecular hydrogen (16, 17). [Se]FDH_H is a monomer polypeptide of M_r 80,000 (18). In addition to selenium, the enzyme contains iron and a molybdopterin guanine dinucleotide cofactor (18).

The selenocysteine of [Se]FDH_H has been replaced with cysteine by site-directed mutagenesis (8), and the resulting mutant enzyme ([S]FDH_H) provides a sulfur analog of a selenoprotein. We report here some of the physical and kinetic properties of this mutant enzyme and compare these properties with those of the wild-type, selenium-containing enzyme.

MATERIALS AND METHODS

Enzyme Purification. The mutant enzyme was produced by *E. coli* strain WL31153 containing the plasmid pFM201. Isolation of the host strain and construction of the plasmid

Abbreviations: [Se]FDH_H, the selenium-containing formate dehydrogenase component of the formate hydrogen lyase complex; [S]FDH_H, the analog of [Se]FDH_H in which the selenocysteine is replaced by cysteine; BV, benzyl viologen; $(V/K)^D$, the deuterium isotope effect on V_{max}/K_m.

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were described by Zinoni *et al.* (8). The organism was cultured as described previously (18). WL31153 is a mutant strain (genotype Δ*selAB*, previously described as Δ*fdhA*) unable to insert selenocysteine into polypeptides. Plasmid pFM201 contains a form of the *fdhF* gene that has been changed by site-directed mutagenesis such that the TGA (selenocysteine) codon has been converted to TGC (cysteine). The mutant enzyme, [S]FDH_H, was purified by the method previously described for the purification of [Se]-FDH_H (18). All procedures for enzyme purification and characterization were performed in the National Institutes of Health Anaerobic Laboratory (19).

Enzyme Assay. Enzyme activity was determined (18, 20) as the formate-dependent reduction of benzyl viologen (BV). The standard assay solution, 1 ml, consists of 20 mM sodium formate, 2 mM BV, and 50 mM Tris·HCl, pH 7.5. Approximately 5.1 μg of [S]FDH_H was used in each assay, unless otherwise noted. One unit of formate dehydrogenase activity is defined as the reduction of 1 μmol of BV per min at 24°C, determined by continuously following the increase in absorbance at 578 nm and using ε_{578} = 11,000 $M^{-1}\cdot cm^{-1}$. In the indicated reactions, deuterioformate (99 atom % deuterium from MSD Isotopes, Merck) replaced formate in the assay solution.

Dependence on pH of Enzyme Activity and Stability. The effect of pH on enzyme activity was determined by measuring the velocity of the reaction catalyzed by [S]FDH_H in solutions that contained 20 mM sodium formate, 2 mM BV, and 25 mM each of sodium phosphate and sodium citrate, adjusted to pH values varying from 4.0 to 8.5.

The effect of pH on stability of the [S]FDH_H enzyme was determined by mixing 10 μl (5.1 μg) of enzyme with 40 μl of storage solutions containing 25 mM sodium sulfate, 2 mM sodium azide, and 93 mM each of sodium citrate and sodium phosphate, adjusted to pH values varying from 4.0 to 8.5. The anaerobic enzyme mixtures in the storage solutions were incubated at 24°C for 3.5 hr, at which time the remaining activity was determined.

Inactivation with Iodoacetamide. To determine the time course of inactivation of [Se]FDH_H by alkylation at pH 6.0, approximately 1.0 μg of enzyme was incubated at 24°C in a solution of 25 mM 2-(*N*-morpholino)ethanesulfonic acid (Mes) at pH 6.0, 20 mM sodium formate, and 20 mM iodoacetamide in a total volume of 100 μl. To determine the rate of inactivation of [S]FDH_H at pH 8.0, 5.1 μg of enzyme was incubated in a solution containing 80 mM sodium citrate, 80 mM sodium phosphate, 20 mM sodium formate, 20 mM iodoacetamide, and 4 mM sodium azide in a total volume of 50 μl at pH 8.0. In both experiments, 10-μl aliquots were removed at the indicated times and immediately mixed into 1 ml of assay solution for determination of remaining activity.

The pH dependence of inactivation by iodoacetamide was determined by incubation of 1.5 μg of [S]FDH_H or 1.5 μg of [Se]FDH_H in 25 μl of 20 mM iodoacetamide, 20 mM sodium formate, 4 mM sodium azide, 80 mM sodium phosphate, and 80 mM sodium citrate, adjusted to pH values varying from 4.5 to 8.5. After 15 min at 24°C remaining activity was assayed. Control incubations were identical except iodoacetamide was not included.

Kinetics. The steady-state rate equation for the reaction is as follows:

$$\frac{1}{v} = \frac{1}{[E]_o}\left(\frac{k_{-1}+k_2}{k_1k_2[A]} + \frac{k_{-3}k_5k_6 + k_4k_5k_6 + k_3k_4k_{-5} + k_3k_4k_6}{k_3k_4k_5k_6[B]} + \frac{k_4k_6 + k_2k_6 + k_2k_4}{k_2k_4k_6}\right), \quad [1]$$

where $[E]_o$, [A], and [B] are the respective concentrations of enzyme, formate, and BV. Descriptions of the reaction and the rate constants involved in the rate equation are found in *Discussion*.

The dissociation constant, K_d, for formate was estimated from the formate concentration at the intersection of the double-reciprocal plots for protio- and deuterio-formate at equal BV concentrations (20). The formate oxidation step rate constant, k_2, was determined from the slopes of the double-reciprocal plots, using the enzyme concentration $[E]_o$, the estimated value of formate K_d, and the assumption $k_2 << k_{-1}$. Values for the BV reduction step rate constants, k_4 and k_6, were estimated by using the equation for k_{cat} (20):

$$\frac{1}{k_{cat}} = \frac{1}{k_2} + \frac{1}{k_4} + \frac{1}{k_6}. \quad [2]$$

RESULTS

Purification. The mutant enzyme was produced and isolated by the same procedure developed for purification of wild-type enzyme (18). The elution profiles of the mutant enzyme from the employed column matrices were nearly identical to those of the wild-type enzyme.

Dependence on pH of Enzyme Activity and Stability. The velocity of formate-dependent reduction of BV catalyzed by [S]FDH_H was determined over a range of pH values, and the results are shown in Fig. 1. Enzyme stability as a function of pH also was determined (Fig. 1). Enzyme stability was determined by measuring the activity remaining after storage of the enzyme for several hours at a given pH value. Also shown in the figure are the corresponding activity and stability profiles for the selenium-containing enzyme (taken from ref. 18). Both profiles for [S]FDH_H are similar to those for [Se]FDH_H, although the [S]FDH_H curves are shifted slightly toward more alkaline pH values.

Inactivation with Iodoacetamide. Both [S]FDH_H and [Se]-FDH_H were inactivated by reaction with the alkylating agent iodoacetamide. Moreover, this iodoacetamide-dependent loss of activity occurred only when formate was present. The requirement for formate in the iodoacetamide-dependent inactivation of [Se]FDH_H at pH 6.0 is illustrated in Fig. 2. Formate was also required for iodoacetamide-dependent inactivation of [S]FDH_H at pH 8.0. Formate alone destabi-

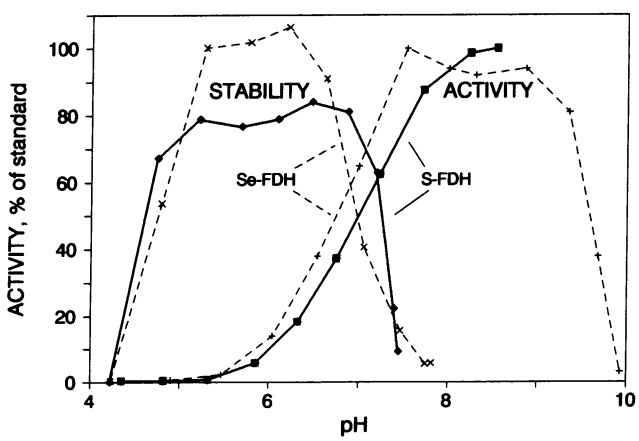


FIG. 1. Stability and activity of [S]FDH_H as a function of pH. The solid lines give the results for experiments performed with [S]FDH_H, while the broken lines give data for [Se]FDH_H previously published in ref. 18. Stability (×, ◆): the activity remaining after incubation for 3.5 hr at the indicated pH values was determined and expressed here as a percentage of initial activity. Activity (+, ■): formate-dependent reduction of BV was assayed at the indicated pH values and expressed here as a percentage of the highest activity.

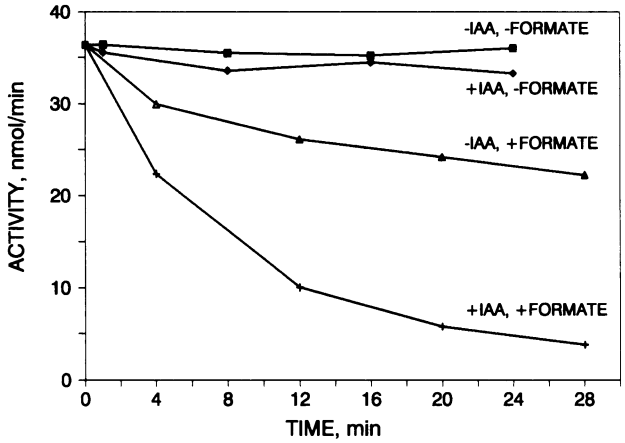


FIG. 2. Time course for iodoacetamide-dependent inactivation of [Se]FDH_H at pH 6.0. Purified [Se]FDH_H was incubated, and remaining enzyme activities were determined at the indicated time points. Enzyme was incubated in the presence of the following: ■, no-additions control; ◇, 20 mM iodoacetamide (IAA); △, 20 mM sodium formate; +, 20 mM iodoacetamide plus 20 mM sodium formate. Azide was not added to these incubations.

lized the enzymes, particularly at pH values above 6.0. However, formate-induced instability was greatly lessened by the presence of azide (data not shown), similar to the protection by azide against oxygen inactivation (18). Reduction of the enzyme with other reducing agents was not studied because carryover interfered in the dye-reduction assay.

Alkylating agents, such as iodoacetamide, react selectively with ionized selenol and thiol groups by nucleophilic displacement. Therefore, iodoacetamide-dependent inactivations were carried out as a function of pH for both [S]FDH_H and [Se]FDH_H, and the results are shown in Fig. 3. Enzyme inactivation by alkylation with iodoacetamide required higher pH conditions for the sulfur enzyme (ionized —SH) than for the selenium enzyme (ionized —SeH).

Kinetic Properties. Activity of the enzyme was determined with 250 mM sodium chloride or 1 mM sodium azide or 50 mM sodium nitrate included in the assay solution. Under these conditions, [S]FDH_H activity was 47%, 27%, or 45%, respectively, the value of activity measured in the absence of these agents.

Enzyme activity was determined as a function of formate and BV concentrations. Double-reciprocal plots of the results (Fig. 4*A*) give a series of parallel lines, indicative of a ping-pong bi-bi kinetic reaction mechanism. Secondary plots of these data give K_m values for formate as 4.9 mM (Fig. 4*B*) and for BV as 2.0 mM (not shown). The V_{max} at saturating concentrations of both substrates was determined (Fig. 4*C*), and from this the turnover number, k_{cat}, was calculated to be 9 sec^{-1}.

Formate was replaced in the activity assay with the isotopic analog deuterioformate. Activity of [S]FDH_H was determined at a variety of concentrations of deuterioformate and BV, and these results appear in Fig. 4*A* for comparison to the results with (protio)formate as substrate. Substitution of deuteriofor-

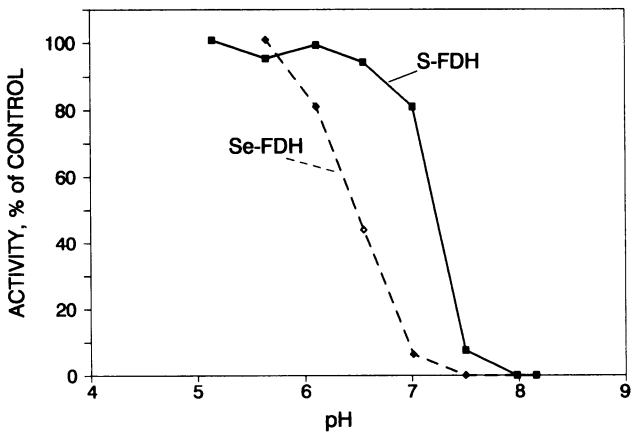


FIG. 3. Iodoacetamide-dependent inactivation of [S]FDH_H and [Se]FDH_H as a function of pH. The enzymes were incubated for 15 min at the indicated pH in solutions containing 20 mM formate and 20 mM iodoacetamide, and activity remaining at the end of this incubation was determined. Details of incubation conditions and activity determinations are given in the text. Remaining activity is expressed here as a percentage of control enzyme samples, which were identical except iodoacetamide was not included. All reaction mixtures contained 4 mM sodium azide.

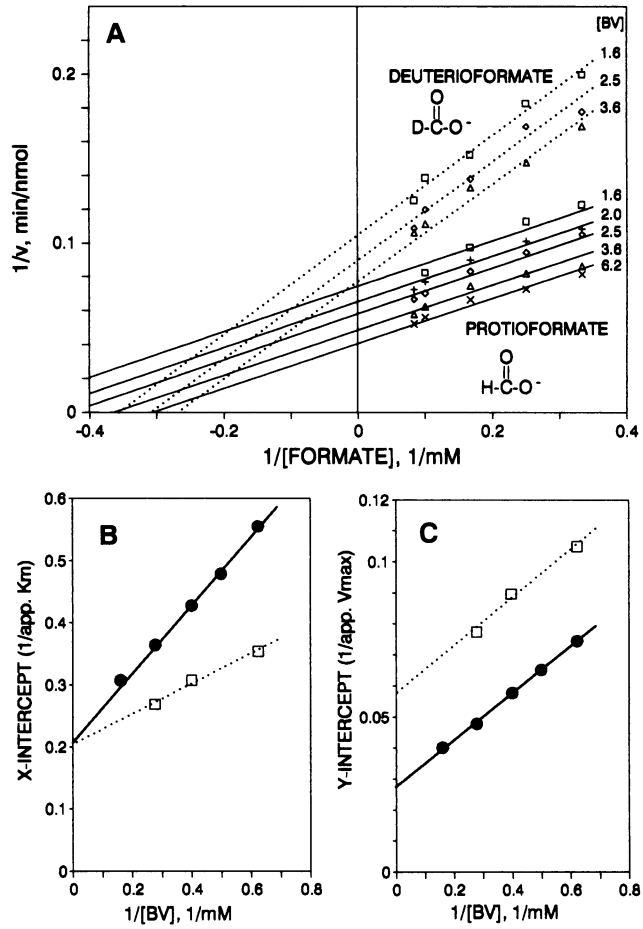


FIG. 4. Kinetics plots for varying formate at different concentrations of BV. [S]FDH_H activity was measured with either protioformate or deuterioformate as substrate. Concentrations of both formate types were varied at the indicated levels of BV, and enzyme activities were determined. (*A*) Double-reciprocal plots. Each line represents the results of analyses performed at the indicated BV concentration: □, 1.6 mM; +, 2.0 mM; ◇, 2.5 mM; △, 3.6 mM; ×, 6.2 mM. Solid lines represent analyses with protioformate as substrate, while dotted lines are for analyses with deuterioformate. The results with protioformate as substrate are the averages of two separate determinations. (*B*) Determination of formate K_m. The *x*-intercepts of the lines in *A* give apparent K_m values at the indicated BV concentrations. These apparent K_m values for protioformate (●) and deuterioformate (□) were plotted against reciprocal BV concentrations. The *y*-intercept of this secondary plot gives the reciprocal of the true K_m at saturating levels of BV. (*C*) Secondary plot for determination of V_{max}. The *y*-intercept (1/apparent V_{max}) values of the lines in *A* were plotted against reciprocal BV concentration. The *y*-intercepts of this secondary plot give reciprocal V_{max} values for protioformate (●) and deuterioformate (□) at saturating levels of formate and BV substrates.

Table 1. Kinetic constants for [Se]FDH_H and [S]FDH_H

Enzyme	k_{cat}, sec^{-1}	k_2, sec^{-1}	k_4, k_6, sec^{-1}	K_m formate, mM	K_d formate, mM	K_m BV, mM	$(V/K)^D$	Ref.
[Se]FDH_H	2,800	11,000 (2,500)*	7500†	26	100	3	4.2	20
[S]FDH_H	9	9.2 (4.6)*	830†	9	5	2	2.2	This work

$(V/K)^D$, the deuterium isotope effect on V_{max}/K_m.
*Numbers in parentheses are values with deuterioformate as substrate.
†Assumes $k_4 = k_6$.

mate for protioformate does not affect K_m (Fig. 4*B*) but greatly reduces the V_{max} (Fig. 4*C*) of the [S]FDH_H enzyme.

DISCUSSION

We have purified and characterized a mutant form of *E. coli* formate dehydrogenase H in which the selenocysteine has been replaced with cysteine. Behavior of the mutant enzyme during purification on hydrophobic interaction and hydroxylapatite chromatography was identical to the behavior of the selenium-containing wild-type enzyme. Thus, the mutant enzyme retains the overall structural properties of the wild-type enzyme.

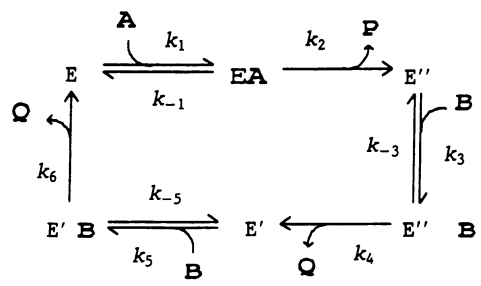
[S]FDH_H and [Se]FDH_H exhibited similar, but not identical, pH profiles for enzyme activity and stability. For [S]FDH_H these pH-dependence curves are shifted slightly toward more alkaline pH values (Fig. 1). The slight shift in activity and stability curves may be related to the replacement of the selenol with the higher-pK_a thiol in the active site; however, the shift alone does not appear sufficient to explain the evolutionary conservation of selenocysteine at the active site of this enzyme.

Only the unprotonated, ionized, forms of selenols and thiols react with iodoacetamide, and consequently the pH dependence of ionization determines the pH dependence of alkylation (21). The pH profiles of alkylation-dependent inactivation (Fig. 3) for [Se]FDH_H and [S]FDH_H suggest that the ionization states of the respective selenol and thiol groups in the proteins are vastly different at pH values near neutrality.

Regardless of pH, neither form of the isolated enzyme was inactivated by treatment with iodoacetamide, under the conditions of the present experiments, unless formate was added. Similarly, numerous preparations of the selenoenzyme glutathione peroxidase were reported to be inactivated with alkylating agent only in the presence of its reducing substrate, glutathione (22, 23). After reaction with formate [Se]FDH_H is highly unstable, possibly due to the formation of reduced, highly reactive redox groups in the enzyme. The selenol group of [Se]FDH_H (and also the thiol group of [S]FDH_H) may be covalently modified in the enzyme(s) as isolated and thus not available for alkylation until reaction with substrate.

[S]FDH_H operates by the same ping-pong bi-bi kinetic mechanism as established previously for [Se]FDH_H (20). Inhibition of [S]FDH_H by compounds such as sodium chloride, azide, and nitrate was similar to that observed with [Se]FDH_H (18). [S]FDH_H therefore appears to catalyze the formate dehydrogenase reaction by a chemical mechanism similar to that of [Se]FDH_H.

The following diagram (20) represents the kinetic steps of the reaction catalyzed by [Se]FDH_H or [S]FDH_H:



In this diagram, the enzyme (E) binds formate (A) to form the enzyme–substrate complex (EA). Formate is oxidized to carbon dioxide (P) concomitant with formation of 2-electron-reduced enzyme (E''). In subsequent steps, one-electron reductions convert oxidized BV (B) to reduced BV (Q).

Kinetic parameters that define [S]FDH_H can be derived from the data presented here. Deuterioformate is a formate analog in which a carbon-deuterium bond replaces the carbon-hydrogen bond. The use of deuterioformate as an alternative substrate has allowed a dissection of the reaction kinetics, such that we can estimate the values for the formate dissociation constant (K_d), as well as rate constants for the formate oxidation step (k_2) and the BV reduction steps (k_4 and k_6). Table 1 presents a comparison of kinetic constants for [S]FDH_H and [Se]FDH_H.

The maximal turnover number (k_{cat}) displays the most notable difference between the two enzymes: the k_{cat} for [Se]FDH_H is 300 times greater than that of [S]FDH_H. The diminished catalytic activity is due to a rate of formate oxidation (k_2) that is three orders of magnitude lower for [S]FDH_H. The replacement of selenocysteine with cysteine in [S]FDH_H caused a diminishment of k_2 such that this step becomes rate-limiting in [S]FDH_H, which is not the case for [Se]FDH_H. These results demonstrate that the selenium of [Se]FDH_H is directly involved in the formate oxidation step, and sulfur is much less effective in this capacity.

Preliminary comparisons of the apparent relative activities of [Se]FDH_H and [S]FDH_H indicated the native selenium enzyme to be 10 to 20 times more active than the sulfur form (ref. 8 and data not shown). These initial studies used crude extracts and partially purified enzyme preparations, the enzyme levels were related on the basis of antibody titers, and activities were determined at single substrate concentrations. As reported here, much greater differences exist in the catalytic rate constants for the purified enzyme under conditions of saturating substrate concentrations.

Both the formate K_m and the formate K_d were greatly reduced in [S]FDH_H as compared with [Se]FDH_H. The K_m for BV was essentially the same for [Se]FDH_H and [S]FDH_H, as shown in Table 1. The significantly lower values for K_m and K_d demonstrate that [S]FDH_H binds formate more tightly than does [Se]FDH_H.

In the case where the value of k_2 is much less than k_{-1}, k_4, and k_6, the equation that defines the formate K_m reduces to

$$K_m(A) = \frac{k_4k_6(k_{-1} + k_2)}{k_1(k_2k_4 + k_2k_6 + k_4k_6)} = \frac{k_{-1}}{k_1} = K_d. \quad [3]$$

Under the conditions of a greatly diminished k_2, we would expect K_m to equal K_d. This is precisely the case observed for [S]FDH_H.

The ratio of V_{max}/K_m values with protio- and deuterio-substrates is known as the deuterium isotope effect on V_{max}/K_m. For [S]FDH_H the $(V/K)^D$ was found to be 2.2. This isotope effect is about half that found for [Se]FDH_H (20). The observed $(V/K)^D$ is equivalent to the isotope effect on k_2 for both [Se]FDH_H and [S]FDH_H.

When [S]FDH_H reaction velocities were determined at formate concentrations greater than 20 mM, the resulting velocities (data not shown) were found to be higher than expected from extrapolation of the results shown in Fig. 4*A*. We cannot definitively explain this anomaly. However, one possible explanation is that very low levels of [Se]FDH_H are present in the [S]FDH_H, and this activity would be apparent only at high formate concentrations due to the higher K_m of the [Se]FDH_H. It has been shown previously that unfractionated *E. coli* aminoacyl-tRNA synthetase preparations will charge cysteyl-tRNA with selenocysteine under certain conditions, although at much reduced rates relative to aminoacylation with cysteine (24). Thus, although the host *E. coli* strain WL31153 does not allow detectable incorporation of ^{75}Se into either the wild-type [Se]FDH_H or the mutant [S]FDH_H (8, 25), it is possible that a low but finite level of nonspecific replacement of cysteine with selenocysteine could have occurred. However, the results of this paper clearly demonstrate that [S]FDH_H displays kinetic and physical characteristics distinct from those of [Se]FDH_H.

The mutant protein differs from wild type at only a single atom, and this atom plays a major role in the catalytic activity of the enzyme. As selenium and sulfur differ by a single filled electron orbital, they share many physical characteristics and reactivities. Differences in the chemical properties of the two elements must account for the large difference in catalytic activity seen between the selenium-containing enzyme and the mutant. The inability of sulfur to replace selenium in an effective manner at this catalytic center helps to explain the evolutionary conservation of selenocysteine as an essential protein component.

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1. Böck, A. & Stadtman, T. C. (1988) *Biofactors* **1**, 245–250.
2. Axley, M. J. & Stadtman, T. C. (1989) *Annu. Rev. Nutr.* **9**, 127–137.
3. Stadtman, T. C. (1990) *Annu. Rev. Biochem.* **59**, 111–127.
4. Berry, M. J., Banu, L. & Larsen, P. R. (1991) *Nature (London)* **349**, 438–440.
5. Cone, J. E., del Rio, R. M., Davis, J. D. & Stadtman, T. C. (1976) *Proc. Natl. Acad. Sci. USA* **73**, 2659–2663.
6. Zinoni, F., Birkmann, A., Stadtman, T. C. & Böck, A. (1986) *Proc. Natl. Acad. Sci. USA* **83**, 4650–4654.
7. Chambers, I., Frampton, I., Goldfarb, P., Affara, N., McBain, W. & Harrison, P. R. (1986) *EMBO J.* **5**, 1221–1227.
8. Zinoni, F., Birkmann, A., Leinfelder, W. & Böck, A. (1987) *Proc. Natl. Acad. Sci. USA* **84**, 3156–3160.
9. Leinfelder, W., Zehelein, E., Mandrand-Berthelot, M.-A. & Böck, A. (1988) *Nature (London)* **331**, 723–725.
10. Zinoni, F., Heider, J. & Böck, A. (1990) *Proc. Natl. Acad. Sci. USA* **87**, 4660–4664.
11. Söll, D. (1988) *Nature (London)* **331**, 662–663.
12. Wu, Z.-P. & Hilvert, D. (1989) *J. Am. Chem. Soc.* **111**, 4513–4514.
13. Wu, Z.-P. & Hilvert, D. (1990) *J. Am. Chem. Soc.* **112**, 5647–5648.
14. Oikawa, T., Esaki, N., Tanaka, H. & Soda, R. (1991) *Proc. Natl. Acad. Sci. USA* **88**, 3057–3059.
15. Peck, H. D. & Gest, H. (1957) *J. Bacteriol.* **73**, 706–721.
16. Stephenson, M. & Stickland, L. H. (1932) *Biochem. J. (London)* **26**, 712–724.
17. Lin, E. C. C. & Kuritzkes, D. R. (1987) in *Escherichia coli and Salmonella typhimurium*, eds. Neidhardt, F. C., Ingraham, J. L., Low, K. B., Magasanik, B., Schaechter, M. & Umbarger, H. E. (Am. Soc. Microbiol., Washington), Vol. 1, pp. 201–205.
18. Axley, M. J., Grahame, D. A. & Stadtman, T. C. (1990) *J. Biol. Chem.* **265**, 18213–18218.
19. Poston, J. M., Stadtman, T. C. & Stadtman, E. R. (1971) *Methods Enzymol.* **22**, 49–54.
20. Axley, M. J. & Grahame, D. A. (1991) *J. Biol. Chem.* **266**, 13731–13736.
21. Huber, R. E. & Criddle, R. S. (1967) *Arch. Biochem. Biophys.* **122**, 164–173.
22. Forstrom, J. W., Zakowski, J. J. & Tappel, A. L. (1978) *Biochemistry* **17**, 2639–2644.
23. Flohe, L. & Guenzler, W. A. (1974) in *Glutathione*, eds. Flohe, L., Benoehr, H. C., Sies, H., Waller, H. D. & Wendel, A. (Thieme, Stuttgart, F.R.G.), pp. 132–145.
24. Young, P. A. & Kaiser, I. I. (1975) *Arch. Biochem. Biophys.* **171**, 483–489.
25. Leinfelder, W., Forchhammer, K., Zinoni, F., Sawers, G., Mandrand-Berthelot, M.-A. & Böck, A. (1988) *J. Bacteriol.* **170**, 540–546.