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Redox Titrations of Carbon Monoxide Dehydrogenase from *Clostridium thermoaceticum*†

Woonsup Shin, Philip R. Stafford,‡ and Paul A. Lindahl*
Department of Chemistry, Texas A&M University, College Station, Texas 77843
Received January 21, 1992; Revised Manuscript Received April 8, 1992

ABSTRACT: Redox titrations of carbon monoxide dehydrogenase (CODH) from *Clostridium thermoaceticum* were performed using the reductant CO and the oxidant thionin. Titrations were followed at 420 nm, a wavelength sensitive to redox changes of the iron–sulfur clusters in the enzyme. When CODH was oxidized by just enough thionin to maximize A_{420}, two molecules of CO per mole of CODH dimer (4 equiv/mol) reduced the enzyme fully. Likewise, 4 equiv/mol of thionin oxidized the fully-reduced enzyme to the point where A_{420} maximized. The four $n = 1$ redox sites which titrated in this region were designated *group I* sites. They include at least two iron–sulfur clusters, $[Fe/S]_A$ and $[Fe/S]_B$, and two other sites, A′ and B′. The $[Fe_4S_4]^{2+/1+}$ cluster in CODH is included in this group. $[Fe/S]_B$ and B′ have reduction potentials (at pH 8) below −480 mV vs NHE; $[Fe/S]_A$ and A′ have reduction potentials above that value. The reduction potential of either $[Fe/S]_B$ or B′ is near to the CO/CO_2 couple at pH 8 (−622 mV). When CODH was oxidized by more than enough thionin to maximize A_{420}, some of the excess thionin oxidized the so-called *group II* redox sites. These sites have reduction potentials more positive than group I and do not exhibit changes at 420 nm when titrated. Titration of group II sites required 1–2 equiv/mol. EPR of reduced group II sites exhibited the $g_{av} = 1.82$ signal. When these sites were oxidized, the only signal present had g values at 2.075, 2.036, and 1.983. The $g_{av} = 1.82$ species either is not an iron–sulfur cluster or is one with unusual optical and redox properties. Thionin in large molar amounts slowly inactivated the enzyme by an oxidation process. Exposure of 100 equiv/mol of thionin to CODH for 1 week completely inactivated the enzyme. These so-called *group III* oxidations are not involved in the catalytic mechanism of the enzyme.

Carbon monoxide dehydrogenase (CODH) is the central enzyme in the acetyl coenzyme A (acetyl-CoA)[1] or Wood pathway [see Ragsdale et al. (1988) for a review], an autotrophic pathway used by acetogenic bacteria to obtain cellular carbon from CO_2. CODH catalyzes the synthesis of acetyl-CoA from CO, coenzyme A, and a methyl group, as well as the reversible oxidation of CO to CO_2 (Ragsdale & Wood, 1985; Lu et al., 1990). The enzyme has an $(\alpha\beta)_3$ quaternary structure and a molecular weight of 440 000 (Ragsdale et al., 1983).

CODH is one of only four enzymes known to contain nickel (Lancaster, 1988). Each $\alpha\beta$ dimer is believed to contain 2 nickels, 11–13 irons, and ~14 acid-labile sulfides (Ragsdale

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* To whom correspondence should be addressed.
‡ Present address: Department of Chemistry, University of Illinois, Urbana, IL.

[1] Abbreviations: CoA, coenzyme A; NHE, normal hydrogen electrode; EPR, electron paramagnetic resonance; EXAFS, extended X-ray absorption fine structure spectroscopy; ENDOR, electron nuclear double magnetic resonance; THF, tetrahydrofuran; SDS, sodium dodecyl sulfate; PAGE, polyacrylamide gel electrophoresis; FCI, ferrous component I.

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et al., 1983) which are arranged into various complexes and clusters. Each dimer also contains ~17 cysteine residues, many of which can be oxidized to form disulfide bonds (Pezacka & Wood, 1986, 1988).

At least one of the nickels and three or four of the irons in the enzyme are part of a novel complex which binds CO. The structure of this so-called *NiFeC complex* has been investigated by EPR (Ragsdale et al., 1982, 1983b, 1985; Lindahl et al., 1990a), EXAFS (Cramer et al., 1987; Bastian et al., 1988), Mossbauer (Lindahl et al., 1990b), and ENDOR (Fan et al., 1991) spectroscopies. Only one model has been proposed that is consistent with all investigations so far: a mononuclear $Ni^{2+/1+}$ complex coordinated mainly by sulfur ligands and chemically linked to an $[Fe_4S_4]^{2+}$ cluster (Bastian et al., 1988; Lindahl et al., 1990b; Fan et al., 1991). In the oxidized state, the NiFeC complex is diamagnetic. It can be reduced by the substrate CO (Ragsdale et al., 1982, 1983b) or by electrochemically-reduced dyes in the presence of CO_2 (Lindahl et al., 1990a). In the reduced state, the complex has an $S = {}^1/_2$ spin state and yields an axial EPR signal at $g = 2.08$ and $g = 2.02$. This half-integer change in spin state indicates that reduction involves a single electron. It is likely that this electron reduces the Ni^{2+} ion of the NiFeC complex rather than the $[Fe_4S_4]^{2+}$ moiety (Lindahl et al., 1990b). The reduction potential for this complex has been estimated at −540 mV vs NHE[2] (Lu et al., 1990; Gorst & Ragsdale, 1991).

The enzyme contains at least one independent $[Fe_4S_4]^{2+}$ cluster. In the oxidized state, the cluster is diamagnetic while the reduced form has a half-integer spin state and yields two EPR signals at $g_{av} = 1.94$ as well as a distinctive Mössbauer component known as *ferrous component I* (Lindahl et al., 1990a,b). The reduction potential for this couple is estimated at −440 mV. Also in the enzyme is an iron-containing species characterized in its reduced state by a rhombic EPR signal centered at $g_{av} = 1.82$ (Lindahl et al., 1990a). Since the species is diamagnetic when oxidized, a one-electron redox reaction is indicated. The reduction potential for this process has been estimated at −220 mV. The signal disappears at potentials below −450 mV and is replaced by another with an average g value of 1.86. It is not known whether this transformation involves electron-transfer reactions or protein conformational changes. The only other metal-containing species that has been characterized in CODH exhibits a Mössbauer component known as *ferrous component II* (FCII) (Lindahl et al., 1990b). The Mössbauer parameters for FCII are typical of a single high-spin ferrous iron coordinated to four sulfur ligands. FCII is redox-active, but no EPR signal has been assigned to it in either of its redox states. FCII may be exchange-coupled to the second Ni ion in CODH, but this is not known with certainty.

One of the most unusual features of the metal complexes just mentioned is the low intensity exhibited by their EPR signals. Values between 0.2 and 0.4 spins/$\alpha\beta$ for each signal (NiFeC, $g_N = 1.94$, $g_B = 1.94$, $g_{av} = 1.82$, $g_{av} = 1.86$), rather than the expected values of 1 spin/$\alpha\beta$, have been reported (Lindahl et al., 1990a). The reasons for these low values are not clear. The correct explanation, once uncovered, will impact greatly on the understanding of these complexes.

Recently, progress has been made in modeling the acetyl-CoA synthase reaction catalyzed by CODH using nickel complexes in a sulfur-rich coordination environment (Stavropoulos et al., 1990, 1991). The compounds $[Ni(NS_3^R)Cl](BPh_4)$ [$NS_3^R = N(CH_2CH_2SR)_3$, R = *i*-Pr, *t*-Bu] in THF can be methylated using CH_3MgCl. The methylated species react with CO to produce acyl derivatives, with react in turn with thiols to yield thioesters and two-electron-reduced forms of nickel. Although these complexes do not act catalytically, they indicate the type of chemistry which may be occurring during the catalytic cycle of the enzyme.

Despite the remarkable progress made in determining the structures and functions of the nickel and iron complexes in CODH, much remains to be learned. The number of complexes and clusters present and their structures, redox chemistry, and magnetic properties are known only to the extent described above. In order to improve our understanding in these areas, we have performed oxidative and reductive titrations of CODH using the reductant CO and the oxidant thionin. In this paper, we report that the redox reactions in CODH are best categorized into three groups. Group I reactions arise from four $n = 1$ redox sites, including the $[Fe_4S_4]^{2+/1+}$ cluster and at least one other iron–sulfur cluster. These sites are reduced by two molecules of CO, and are likely involved in the catalytic mechanism of the enyzme. Group II reactions arise from one or two redox sites, including the $g_{av} = 1.82$ species. These sites have more positive reduction potentials than the group I sites and do not have absorbance properties typical of iron–sulfur clusters. Group III oxidative reactions inactivate the enzyme and are not involved in the catalytic mechanism.

Experimental Procedures

Analytical Methods. Protein concentrations were determined by the biuret and Bradford colorometric methods (Pelley et al., 1978; Sedmak et al., 1977). The concentrations of thionin (3,7-diaminophenothiazin-5-ium chloride, Aldrich) in solutions were determined by titration against a sodium dithionite solution which had itself been standardized against potassium ferricyanide. CO-saturated buffer was prepared by replacing the atmosphere above a solution of anaerobic buffer (50 mM Tris·HCl, pH 8.0) with oxygen-scrubbed (Oxisorb, MG Scientific) research-grade CO (99.99%, Scott Specialty Gases) and stirring the solution vigorously for at least 30 min. The concentration of CO in the buffer was determined by the following procedure: a trace amount of CODH was added to a cuvette containing 50 mM Tris, pH 8.0, and 10 mM oxidized methyl viologen. The amount of methyl viologen reduced after injection of a known volume of the CO-saturated buffer was determined from the absorbance at 604 nm and the extinction coefficient of reduced methyl viologen at this wavelength (1.39×10^4 M^{-1} cm^{-1}). The concentration of CO in the buffer was calculated to be 1.08 mM in one experiment and 1.09 mM in another. These values agree well with that published for the solubility of CO in water at 20 °C (1.03 mM) (Budavari et al., 1989). The average of the two measured values was used as needed in calculations.

Protein Purification and Characterization. Clostridium thermoaceticum was grown essentially as described (Lundie & Drake, 1984), and CODH was purified and assayed according to a modification of published procedures (Ragsdale & Wood, 1985; Ramer et al., 1989). Unless mentioned otherwise, all procedures involving CODH were performed in a glovebox (Vacuum/Atmospheres) containing an argon atmosphere with less than 3 ppm of oxygen. Buffers were rendered oxygen-free using procedures to be described elsewhere.

One thionin titration and two CO titrations (A and B) are presented in this paper. The enzyme used was essentially pure according to SDS–PAGE, with only the α and β subunits present. The enzyme used for the thionin titration and for CO

[2] Potentials quoted in the text are vs NHE.

Table I: Extinction Coefficients (ϵ) of Various Iron–Sulfur Proteins

protein, source	cluster type	wavelength (nm)	ϵ_{ox} (M^{-1} cm^{-1})	ϵ_{red} (M^{-1} cm^{-1})	$\Delta\epsilon_{ox-red}$ (M^{-1} cm^{-1})	reference
CODH, *Clostridium thermoaceticum*		420	45400	30700	14700	this work
ferredoxin, *Clostridium acidi-urici*	$[Fe_4S_4]^{2+/1+}$	390	12250[a]	5400[c]	6850	Mayhew et al. (1969)
ferredoxin, *Clostridium pasteurianum*	$[Fe_4S_4]^{2+/1+}$	390	12250[a]	5600[c]	6650	Mayhew et al. (1969)
ferredoxin, *Peptostreptococcus elsdeni*	$[Fe_4S_4]^{2+/1+}$	390	12250[a]	5800[c]	6450	Mayhew et al. (1969)
ferredoxin II, *Desulfovibrio gigas*	$[Fe_3S_4]^{1+/0}$	415[b]	15700	9600	6100	Bruschi et al. (1976)
ferredoxin, spinach	$[Fe_2S_2]^{2+/1+}$	420	9400	4400[c]	5000	Mayhew et al. (1969)

[a] The extinction coefficient is given on a per cluster basis. [b] The extinction coefficient of the reduced form was measured at 425 nm. [c] This value was estimated from results given in Mayhew et al. (1969).

titration A had a specific activity of 193 units/mg for CO oxidation, and 0.27 unit/mg for carbonyl exchange activity. EPR spectra of this protein, when reduced by CO, exhibited the NiFeC (0.2 spin/$\alpha\beta$), g_{av} = 1.94 and 1.86 signals. The overall intensity in the g = 2 region corresponded to 1.1 spins/$\alpha\beta$. The sample used in CO titration B had essentially the same protein purity and EPR signals as that used in titration A and a somewhat higher specific activity (350 units/mg). However, the significant difference between CO titrations A and B was not the samples per se, but the method by which they were prepared for titration.

Titration Protocol. The procedure used for CO titration A was as follows: sodium dithionite was removed from 35 mg of CODH (125 mg/mL) by passing the solution through a Sephadex G25 (Pharmacia) column (14 cm × 0.7 cm) equilibrated in 50 mM Tris·HCl, pH 8.0. Two-thirds of the eluted protein was oxidized by adding thionin, an oxidant with an intense blue color. The quantity of thionin added, approximately 2 equiv/mol,[3] was *insufficient to turn the color of the protein solution blue.* Thionin was then removed by passing the solution through another Sephadex G25 column (prepared as above). The bottom 3 cm of the column consisted of Sephadex G25 to which thionin had been covalently attached according to a published method (Porath, 1974). Three-quarters of the protein that eluted from this column was used in CO titration A. The same procedure was used for CO titration B except that the sample was oxidized *by adding sufficient thionin for the protein solution to remain blue for approximately 1 min.*

The CO titrations were performed outside of the glovebox in a 5-mL quartz cuvette (Starna, type 17) modified for anaerobic titrations with a double-septum seal (Averill et al., 1978). Aliquots of CO-saturated buffer rather than the gas itself were added during the titration. Cuvettes were filled with protein solution so that only a small bubble (~500 μL) remained. The bubble served in mixing the solution after each addition of CO buffer. This procedure minimized the partitioning of CO between liquid and gas phases, increased the rate of CO reduction, and simplified our calculations. Titrations were monitored by measuring the absorbance (Perkin-Elmer Model λ3B) at 420 nm (A_{420}). Thirty seconds of mixing followed by 30 s for "settling" was used to obtain stable A_{420} readings. Measured A_{420} values were adjusted for dilution and were plotted as $(A_{420} - A_{min})/(A_{max} - A_{min})$ vs the equiv/mol of CO added, where A_{min} and A_{max} were the minimum and maximum absorbances obtained, respectively. At the end of the titration (after adding 8.8 equiv/mol of CO), bubbling CO gas through the samples produced no additional drop in A_{420}. We therefore designated the A_{420} value obtained at the end of the titration to be A_{min}. When more thionin was added to the unused portion of the protein solution that eluted from the derivatized Sephadex G25 column, A_{420} also remained unchanged. Consequently, we designated the A_{420} value obtained by this oxidation procedure to be A_{max}. The $(A_{420} - A_{min})/(A_{max} - A_{min})$ values, multiplied by 100, will be designated as "percent oxidized at 420 nm".

The volumes of the solutions in the cuvette were determined after the titrations from the mass of the solutions, assuming that they had the density of water at 25 °C (0.997 g/mL). Enzyme concentrations were determined from the concentrations of nickel and iron in the samples (using ICP), assuming that each $\alpha\beta$ dimer contained 2 Ni and 13 Fe. For CO titration A, the final volume and nickel, iron, and calculated CODH concentrations were 4.72 mL, 25.2 μM, 160 μM, and 12.5 μM, respectively. For CO titration B, the final volume and nickel and calculated CODH concentrations were 4.84 mL, 18.6 μM, and 9.3 μM, respectively. The specific activities of the samples after CO titrations A and B were 207 and 380 units/mg, respectively.

The thionin titration was performed using essentially the same procedures. One-third of the dithionite-free CODH sample, the preparation of which is described above, was used in the titration. A_{min} was obtained by adding 19 equiv/mol dithionite to the solution after the titration was complete. The final volume and nickel, iron, and calculated CODH concentrations were 5.08 mL, 21.0 μM, 141 μM, and 10.7 μm, respectively. The specific activity of the sample after titration was 212 units/mg.

Sample Preparation for EPR Spectroscopy. Sodium dithionite was removed, using Sephadex G25 chromatography, from a sample of CODH obtained from the same stock used for CO titration A and the thionin titration. Thionin was added to the dithionite-free protein in the double-septum-sealed cuvette until A_{max} was obtained and a *faint* blue color was briefly evident. A sample of the solution was removed and analyzed by EPR spectroscopy. Additional thionin was added so that the solution remained blue for more than 4 min, and another sample was removed for later analysis by EPR. The EPR system consisted of a Bruker ESP300 spectrometer, an Oxford Instruments ER910A cryostat, an HP5352B frequency counter, and a Bruker ER035M NMR gaussmeter.

Results and Discussion

Absorption Spectra of CODH. The absorption spectrum of thionin-oxidized CODH (Figure 1, solid line) exhibited a shoulder in the 400-nm region. Oxidized iron–sulfur clusters of all types exhibit sulfur-to-iron charge-transfer transitions in this region (Orme-Johnson, 1973; Holm & Ibers, 1977; Berg & Holm, 1982), and this shoulder most certainly arises from the iron–sulfur clusters in CODH. The intensity of this shoulder diminished in the spectrum of the CO-reduced enzyme (Figure 1, dashed line), a commonly encountered event when iron–sulfur clusters become reduced. The oxidized-minus-reduced difference spectrum (Figure 1, inset) indicates that the greatest absorption change occurred at 420 nm, so

[3] The equivalents of a reductant or oxidant added per mole of CODH $\alpha\beta$ dimer will be referred to as equiv/mol.

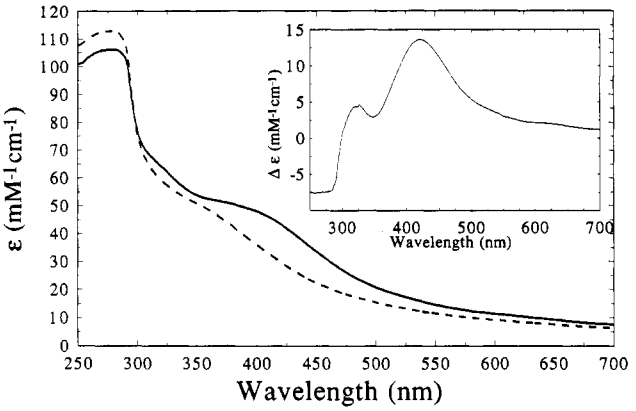

FIGURE 1: Electronic absorption spectra of CODH. The solid line is of CODH after oxidation by thionin. The dashed line is of CODH after addition of 8.9 equiv/mol of CO. The inset is the oxidized-minus-reduced difference spectrum. The sample used to obtain these spectra was that used for CO titration A.

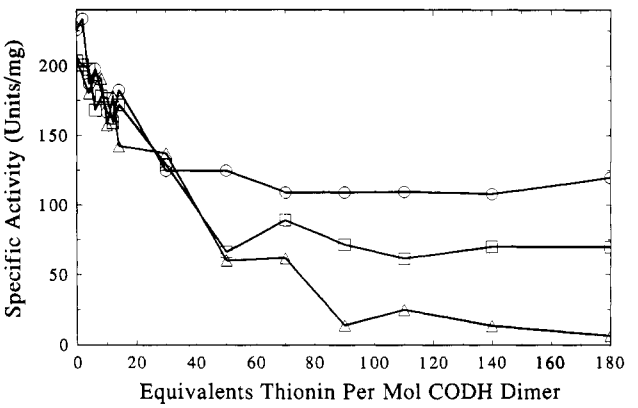

FIGURE 2: Oxidative inactivation of CODH by thionin. Amounts of thionin, ranging from 0 to 180 equiv/mol, were added to 0.84-mg aliquots of CODH in final volumes of 1.5 mL. After 1, 2, and 19 days, samples were assayed for activity (circles, squares, and triangles, respectively), and their colors were noted. Samples with less than or equal to 30, 50, or 90 equiv/mol were brown after 1, 2, and 19 days, respectively.

titrations were monitored at this wavelength. The extinction coefficients of the oxidized and reduced forms of the enzyme and various iron–sulfur clusters are given in Table I.

Oxidative Inactivation of CODH by Group III Reactions. Thionin is a mild oxidant commonly used to oxidize metalloproteins (Fultz & Durst, 1982; Lindahl et al., 1988). It was selected for use with CODH because (1) its reduction potential (+60 mV) was appropriate for titrating the complexes in CODH, (2) it exhibited a distinct color change from blue to colorless upon reduction (measurable at 600 nm) which could be used as an accurate end-point indicator, and (3) it did not exhibit an EPR signal in either of its oxidation states.

Despite these exceptional properties, thionin in large molar amounts was found to slowly inactivate CODH through an oxidative process. This phenomenon is demonstrated by the experiment shown in Figure 2. From 0 to 180 equiv/mol of thionin was added to solutions of CODH. The specific activities and the color of the solutions were determined at different times. The presence of unreacted thionin was indicated by a blue solution; brown solutions, the color of CODH, indicated that all of the added thionin had reacted.

Surprisingly, most of the samples turned brown. In fact, only those containing 140 and 180 equiv of thionin/mol of CODH remained blue for the duration of the experiment (6 weeks). The rate at which these redox reactions occurred declined with increasing amounts of thionin. For example, 1 day was required for the blue color of the 30 equiv/mol sample to disappear, while 2 days were needed for the 50 equiv/mol sample. By day 19, only the 110, 140, and 180 equiv/mol samples remained blue.

To determine whether the reacted thionin was reduced or degraded in this process, samples similar to those which turned brown in the experiment just described were exposed to oxygen. The blue color indicative of oxidized thionin returned with the intensity expected if all of the thionin originally added had reoxidized. This demonstrated that the loss of blue color in these experiments was due to the *reduction* of oxidized thionin. Moreover, since only CODH and (redox inactive) buffer were present in solution, we conclude that CODH was becoming oxidized in this process.

The enzyme activity was adversely affected by these redox reactions. Although thionin was added to each sample on day 0, the activity declined only as the thionin reacted. This can be seen by comparing the activities obtained at 1, 2, and 19 days into the experiment (Figure 2) to the colors of the solutions at those times. Thus, we conclude that thionin slowly inactivates CODH by oxidizing it.

These oxidations cannot be used in the catalytic mechanism of the enzyme; they occurred too slowly, and they inactivated the enzyme. Since our interests were in redox reactions pertinent to the catalytic mechanism of the enzyme, we did not study these reactions further. To distinguish these reactions from others that occur in CODH, they will be referred to as *group III* reactions.

We attempted but were unable to find a suitable alternative to thionin; those with similar or more positive reduction potentials appeared to have similar effects on CODH. Milder oxidants either were incapable of completely oxidizing all of the sites in CODH which might be involved in the catalytic mechanism or had absorption features which precluded in the monitoring of titrations at 420 nm. Fortunately, the deleterious effects of thionin occurred only when large molar amounts were used. In fact, once the existence of the group III reactions was realized, we were able to use their onset as an end-point indicator of another group of redox reactions in CODH (group I) and as a "start-point" indicator of yet another group of redox reactions (group II). However, before the properties of these other groups of reactions can be properly described, another unusual phenomenon of CODH must be mentioned.

"Spontaneous" Oxidation of Reduced CODH. We found that when CODH was more than 50% reduced at 420 nm, it reoxidized in a slow and seemingly spontaneous process (spontaneous in the sense that it occurred without intentionally adding an oxidant). In one experiment, 5 equiv/mol of sodium dithionite was added to oxidized CODH, and the oxidation state of the enzyme was followed at 420 nm in time after the addition (Figure 3). This process required hours to complete and left the enzyme approximately 50% oxidized at 420 nm. We recently discovered that CODH solutions slowly catalyze the reaction $2H^+ + 2e^- \rightleftharpoons H_2$ (Shin and Lindahl, unpublished results) and suggest that reduced CODH "spontaneously" oxidized as protons were reduced to molecular hydrogen. The fact that this process ceased after the enzyme was about 50% oxidized at 420 nm suggests that some of the iron–sulfur clusters in CODH (those with reduction potentials near to or more negative than that of the H^+/H_2 couple at pH 8) reoxidized as they reduced $2H^+$ to H_2 and that other iron–sulfur clusters, in their reduced forms, were unable to carry out this reduction.

During the titrations, we wanted to measure A_{420} values reflecting equilibrium conditions for the reduction of redox

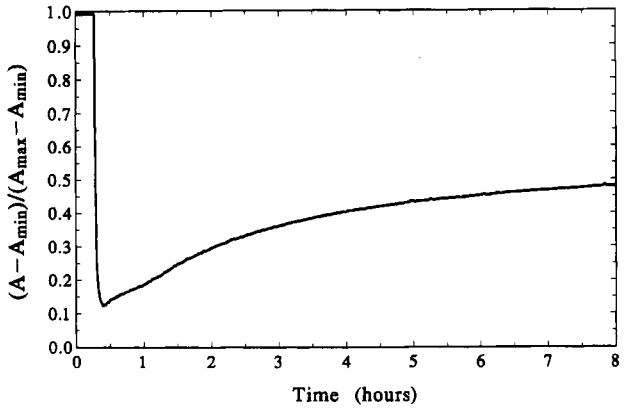

FIGURE 3: "Spontaneous" oxidation of reduced CODH. 5 equiv/mol of sodium dithionite was added to thionin-oxidized CODH. The solid line is the fractional change in absorbance at 420 nm occurring after the dithionite addition.

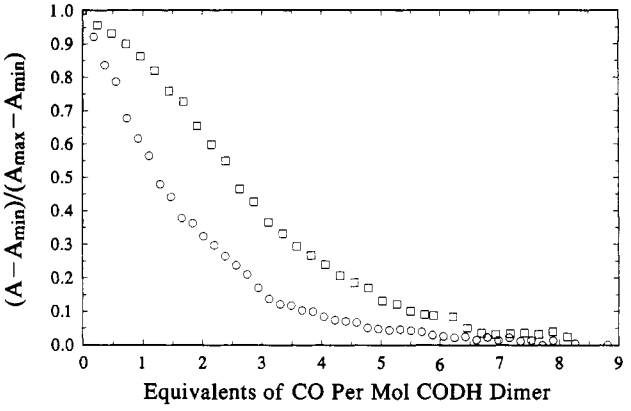

FIGURE 4: CO reductive titrations of CODH. Titrations A (circles) and B (squares) are described in the text. The sample used in A was oxidized by only enough thionin to maximize A_{420}, while additional thionin was used to oxidize the sample used in B.

sites by CO and for their oxidation by thionin. The usual method for achieving such conditions involves measuring absorbance values long enough after adding titrant for the values to cease changing. However, the hydrogenase activity precluded our use of this method here. Nevertheless, such equilibrium conditions could be approximated quite well because the rates of reduction of CODH by dithionite (Figure 3, the rapid decay in A_{420}) and CO (not shown) were about 100–400 times faster then the reoxidation rate of CODH by protons (Figure 3, the slow increase in A_{420}). CO reductions were essentially complete within 1 min after mixing. In contrast, the reoxidation of CODH by protons was only about 10% complete after 20 min. Thus, the strategy which proved successful was to complete the last half of the CO titration, where the hydrogenase activity was operative, in about 20 min. A similar strategy was used for the thionin titration. Using this strategy, "spontaneous" oxidation was not given sufficient time to cause significant changes in the redox state of CODH.

CO Reductive Titrations. The titration curve of CO reductive titration A is shown in Figure 4 (circles). The sample used in this titration was oxidized by just enough thionin to maximize the absorbance at 420 nm. Over 90% of the overall absorbance changes that occurred during the titration were complete after adding 4 equiv/mol of CO. The curvature evident between 3 and 6 equiv/mol of CO probably occurred because the reduction potential of the site undergoing reduction in this region was near that of CO/CO_2 at pH 8 (−622 mV) (Weast, 1985), and it could not be reduced stoichiometrically by CO. We conclude that under the conditions of this experiment, 4 equiv/mol of CO was transferred into CODH before spectral changes at 420 nm ceased. We will refer to the redox reactions that occurred here as *group I* reactions, and the species at which such reactions occurred as *group I* redox sites.

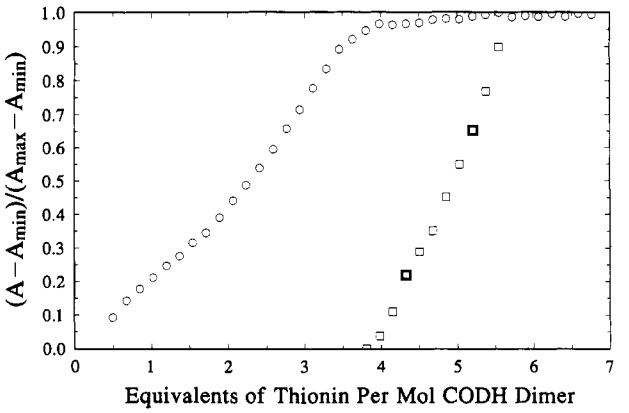

FIGURE 5: Thionin titration of CODH. Circles represent the fractional absorbance changes at 420 nm. Squares represent the number of equiv/mol of thionin added that was used for group III oxidations. The ordinate scale for the squares is in equiv/mol of thionin. Other details are given in the text.

The titration curve exhibited little structure other than a gradually decreasing slope. Such changes in slope indicate that the sites being reduced do not have identical optical properties and did not all change oxidation states simultaneously during the titration. If they had identical optical properties or reduction potentials, the titration curve would have been a straight line with a slope of $-1/n$ mol/equiv, where n is the total number of equiv/mol transferred during the titration.

Thionin Oxidative Titration. Samples used for oxidative titrations should be fully-reduced and reductant-free prior to being titrated, so that the number of equiv/mol of oxidant added relates directly to the number of redox sites in the enzyme. However, samples of CODH from which reductant was removed chromatographically were significantly oxidized by the time their spectra could be obtained. This oxidation occurred because solutions of fully-reduced CODH slowly reduced protons to molecular hydrogen. To circumvent this process, dithionite was added to CODH just prior to commencement of the titration. Just enough was added so that the enzyme was reductant-free and *nearly* fully-reduced. Because the hydrogenase activity was much slower than the oxidation of the enzyme by thionin, we were able to perform the titration without noticeable effects from the hydrogenase activity.

The sample used in the titration was 48% reduced at 420 nm prior to commencement of the titration. After addition of 2.8 equiv/mol of sodium dithionite, the sample was 91% reduced at 420 nm. The titration curve obtained by adding aliquots of thionin to this sample is shown in Figure 5. We estimate that the 91% reduced enyzme was 0.5 equiv/mol oxidized at the beginning of the titration, and so the data have been translated to the right by this amount. Our estimate was based on the following procedure: a straight line was fitted to the first eight data points and extended to the origin of the ordinate. The abscissa was shifted by the amount (0.5 equiv/mol) required for the origins of both axes to intersect.

Over 95% of the overall absorbance changes that occurred during the titration were complete after addition of 4 equiv/mol of thionin. The rather abrupt cessation of changes at 420 nm after addition of 4 equiv/mol suggests that thionin

stoichiometrically oxidized all of the sites whose redox changes were monitored at 420 nm. We conclude that 4 equiv/mol was removed from CODH before spectral changes at 420 nm ceased and that the sites oxidized in this part of the titration were the same (group I) sites reduced during CO titration A. Group I sites can thus be both oxidized and reduced.

The titration curve had some structure, most notably the increase in slope that occurred past 2 equiv/mol. This was further evidence that the sites being oxidized did not have identical optical properties and did not change their oxidation states simultaneously during the titration.

Group I Redox Sites in CODH. We used the cessation of changes in A_{420} as an end-point indicator for the titration of group I sites in CODH. The cessation of spectral changes can be used as an end-point indicator of a stoichiometrically-titratable site when one of the following conditions is met: (1) the species under investigation exhibits *spectral* sensitivity (i.e., its spectral properties change when it changes oxidation state); (2) the species does not exhibit such changes, but it titrates either before or during the titration of another species which does. A species which exhibits no spectral changes when it changes oxidation state cannot be monitored spectrally if it titrates *after* all spectrally-sensitive species. Thus, group I sites must include A_{420}-sensitive species and may include species which exhibit no A_{420} sensitivity but which titrate prior to or during the titration of at least one species which is A_{420}-sensitive.

Due to these relationships, if all of the group I sites titrated in-order, one after another in a potential-dependent fashion, then those with the most positive and most negative reduction potentials would have to be A_{420}-sensitive. The changes observed in the slopes of the CODH titration curves indicate that group I sites do not all have identical optical properties and do not titrate simultaneously. However, it is not known whether all sites titrated in-order. There are many possibilities besides the extreme cases where sites titrate simultaneously or in-order, depending upon the relative reduction potentials of the sites.

The group I sites must include some or all of the iron–sulfur clusters in CODH. The best-characterized iron–sulfur cluster in CODH is the $[Fe_4S_4]^{2+/1+}$ cluster yielding the $g_{av} = 1.94$ signals and the FCI Mössbauer component (Lindahl et al., 1990a,b). These spectral properties, as well as the redox properties of this cluster, are quite typical of $[Fe_4S_4]^{2+/1+}$ clusters generally. Although its UV–vis properties have not been determined, such clusters upon reduction typically experience a drop of about 6600 M^{-1} cm^{-1} in their extinction coefficient in the 400-nm region (Table I). From the changes in the extinction coefficient values observed upon reduction of CODH (14 700 M^{-1} cm^{-1}), we estimate that the reduction of this $[Fe_4S_4]^{2+}$ cluster is responsible for about half of the total absorption changes (6600/14 700) and that the other half arises from the reduction of *at least* one other iron–sulfur cluster. The fact that half of the total A_{420} changes that occurred in CODH resulted when enzyme solutions reduced $2H^+$ to H_2 (Figure 2) indicates that at least one iron–sulfur cluster in CODH has its reduction potential near to or below that of the H^+/H_2 couple at pH 8 (−480 mV) and that at least one other cluster has its reduction potential above −480 mV. We will refer to these two sites as $[Fe/S]_A$ and $[Fe/S]_B$ (where A and B denote above and below, respectively). Since their potentials are quite different, these two sites must titrate in-order.

The thionin titration indicates that about 2 equiv/mol is removed from fully-reduced CODH when the enzyme is 50% oxidized at 420 nm. Since the $[Fe_4S_4]^{2+/1+}$ cluster in CODH undergoes one-electron transfers, and this cluster represents either $[Fe/S]_A$ and $[Fe/S]_B$, there must be at least one other $n = 1$ site, call it A′ or B′, with similar proton-reducing abilities. For example, if the $[Fe_4S_4]^{2+/1+}$ cluster corresponded to $[Fe/S]_B$, then there must be an $n = 1$ site B′.

A priori, the other [Fe/S] cluster (to continue the example, let this cluster be $[Fe/S]_A$) could undergo either one-electron- or two-electron-transfer reactions. We discount the latter possibility on the grounds that all well-characterized iron–sulfur clusters undergo one-electron-transfer reactions (Orme-Johnson, 1973; Holm & Ibers, 1977; Berg & Holm, 1982; Lindahl & Kovacs, 1990) and that the spin-state changes occurring upon oxidation or reduction of every iron-containing cluster in CODH are those expected for $n = 1$ redox reactions (Ragsdale et al., 1982, 1983, 1985; Lindahl et al., 1990a,b). Thus, if $[Fe/S]_A$ undergoes one-electron transfers, there must be a fourth group I site, which we designate *A′* which engages in $n = 1$ redox chemistry as well.

We conclude that there are four group I sites, designated here as $[Fe/S]_A$, $[Fe/S]_B$, A′, and B′. All sites undergo one-electron redox reactions. At least two of these sites are iron–sulfur clusters, including the $[Fe_4S_4]^{2+/1+}$ cluster. $[Fe/S]_B$ and B′ have reduction potentials below that required to reduce protons to molecular hydrogen; $[Fe/S]_A$ and A′ are incapable of effecting that reduction. Finally, since group I sites can be reduced by the substrate CO, we suspect that they are involved in the mechanism used by CODH to catalyze the oxidation of CO to CO_2.

Group II Redox Sites in CODH. We discovered another group of redox reactions which can occur in CODH; we will refer to these as *group II* reactions, and the species from which they arise as *group II* redox sites. The evidence for these sites is as follows.

The titration curve of CO reductive titration B is shown in Figure 4 (squares). The sample used in this titration was oxidized by more than enough thionin to maximize the absorbance at 420 nm. Some of the thionin added in excess oxidized species in CODH which were not oxidized by the procedure used in titration A. When titrations A and B are compared, three differences are evident: (1) the sample used in B required a greater number of equiv/mol of CO for complete reduction; (2) the initial slope of titration B was only 30% of that in A; (3) by horizontally translating titration curve B to the left by 1.5 equiv/mol, the second halves of curves A and B become aligned. Thus, when CODH was oxidized with more thionin than that needed for the group I sites, additional species, which we designate *group II* redox sites, became oxidized. The group II sites were among the first to be reduced by CO, which suggests that they have reduction potentials more positive than the group I sites. The flattened slope in the initial region of titration curve B indicates that the group II sites are not strongly A_{420}-sensitive. The degree to which titration B had to be translated suggests that either 1 or 2 equiv/mol is required to reduce these sites.

These characteristics suggested that during the thionin titration the group II sites ought to have been oxidized after the group I sites. Since the group II sites did not appear to exhibit significant spectral changes at 420 nm upon their reduction, we felt that evidence for their oxidation by thionin might be more forthcoming if oxidized thionin itself was used as an end-point indicator (by measuring A_{600}). For this reason, we desired to measure the point at which residual, unreacted thionin developed during the thionin titration. At issue was whether residual thionin appeared immediately after the group

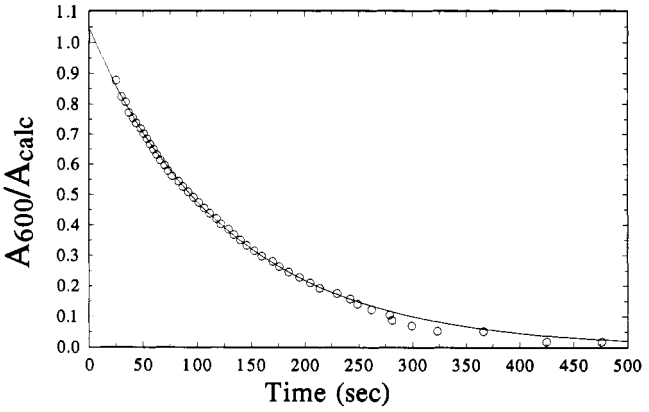

FIGURE 6: Time course of thionin decay due to group III oxidations. The absorbance at 600 nm of a CODH sample already oxidized by 5.90 equiv/mol thionin was measured vs time after the addition of another 0.17 equiv/mol of thionin. The A_{600} value expected from adding the same amount of thionin to an equivalent volume of water (4.66 mL), referred to as A_{calc}, was calculated using the extinction coefficient of thionin at 600 nm (2.80×10^4 N^{-1} cm^{-1}). The experimental A_{600} values were divided by A_{calc}, and the quotients have been plotted vs time in the figure. The solid line is a plot of $A_{600}/A_{calc} = 1.044 \exp(-0.0078t)$, obtained by least-squares-fitting of the data out to 250 s. The value -0.0078 s^{-1} is the negative apparent first-order rate constant for this decay. Such rate constants calculated for every 0.17 equiv/mol injection of thionin declined in proportion to the total amount of thionin in the sample. Values ranged from 0.046 s^{-1} for the point of 3.98 equiv/mol of thionin to 0.007 s^{-1} for the point at 6.77 equiv/mol of thionin. The oridinate intercept for the data shown would equal 1.0 if all of the additional thionin added were reduced by the decay process. For the particular point shown, the deviation from 1 is less than 5%, typical of injections after the 5.5 equiv/mol of thionin point. For points between 4.0 and 5.5 equiv/mol, the intercept values were significantly less than 1 (0.45–0.67) because some of the added thionin was utilized rapidly to oxidize group II sites.

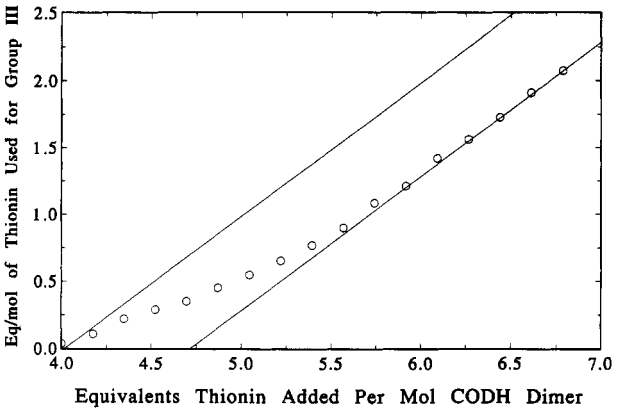

FIGURE 7: Evidence for group II redox sites in CODH. Circles represent the total number of equiv/mol of thionin used in group III oxidations, as determined by the method described in Figure 6, vs the total number of equiv/mol of thionin added during the titration. The solid line on the left indicates where the data would lie if all of the thionin added after the 4.0 equiv/mol point were exclusively used for group III oxidations. The solid line on the right indicates where the data would lie if all of the thionin added after the 4.7 equiv/mol point were exclusively used for group III oxidations.

I sites were oxidized or whether other (group II) sites were oxidized after the group I sites.

The problem with using thionin as an end-point indicator for CODH titrations is that it oxidatively inactivates CODH at some point after it finishes oxidizing the group I redox sites. As described earlier, over 100 equiv/mol of thionin must be added before residual thionin develops. We developed a strategy to circumvent this problem, on the basis of the fact that thionin oxidized group I sites immediately upon mixing, while it oxidatively inactivated CODH at rates slow enough to measure using our instrumental setup. We decided to accurately determine the point beyond which added thionin was used for group III oxidations, to determine whether group III oxidations occurred *immediately* after oxidation of the group I sites, or whether there was another group of sites (group II) which became oxidized after the group I sites and before the onset of group III oxidations.

The amount of thionin used in group III oxidations was determined as follows. For each point in the titration (past 4 equiv/mol), rates of thionin reduction were measured, by monitoring A_{600} (Figure 6). Plots of the natural log of the data vs time fit to straight lines, indicating that group III oxidations occurred by a first-order process. The amount of thionin used for group III oxidations for each injection of thionin was calculated by extrapolating exponential fits of the data back to the time of mixing. The *total* number of equiv/mol of thionin used for group III oxidations up to and including any particular point in the titration was obtained by summing the amounts calculated for each thionin addition up to and including the amount used for that particular point. These totals have been plotted as the squares in Figure 5 (and the circles in Figure 7). The x-axis intercept of the data, at 4 equiv/mol, must be the end-point in the titration of the group I sites, since it occurred at the same point in the titration where spectral changes at A_{420} ceased.

This result indicated that the onset of group III oxidations occurred *immediately* after oxidation of the group I sites. However, a closer inspection of the data revealed that not all of the thionin added in this region was used to oxidatively inactivate CODH. Figure 7 shows a plot of the equiv/mol of thionin used for group III oxidations vs the equiv/mol of thionin added. The solid line on the left indicates where the data would have fallen if all of the added thionin had been used for group III oxidations. The line of the right has the same slope, but is displaced by 0.7 equiv/mol. The data past 5.5 equiv/mol followed the displaced solid line, indicating that after 5.5 equiv/mol of thionin was added, *all* of the added thionin was used for group III oxidations. The data between 4.0 and 5.5 equiv/mol did not follow the line, indicating that, in this region, some of the added thionin (0.7 equiv/mol) was used to oxidize another group of sites (group II). This other group exhibited virtually no A_{420} changes when oxidized and titrated after the group I sites. These optical and redox properties were similar to those displayed by the group II sites reduced at the beginning of CO titration B, and we suggest that they are, in fact, the same sites.

Thus, group II sites were oxidized during the same region of the thionin titration in which group III oxidations occurred. Also, the group II and some group I sites were reduced during the same region of CO titration B. These overlapping redox processes precluded the detailed study of the group II sites. To identify them more specifically, EPR spectra were obtained of samples prepared under conditions where the group II sites were reduced (Figure 8A) and oxidized (Figure 8B). The spectrum of the reduced sites was dominated by the $g_{av} = 1.82$ signal, while that of the oxidized sites contained only a signal with g values at 2.075, 2.036, and 1.983. This latter signal has not been reported before, and we will refer to it as the $g_{av} = 2.03$ signal. Inspection of Figure 8A reveals that, besides the $g_{av} = 1.82$ signal, a small amount of the $g_{av} = 2.03$ signal was also present. The most significant result of this experiment is that oxidation of group II sites caused the $g_{av} = 1.82$ signal to disappear; this means that the $g_{av} = 1.82$ species is one of the group II sites. This species is known to contain at least two irons (Lindahl et al., 1990b). The fact that the oxidation

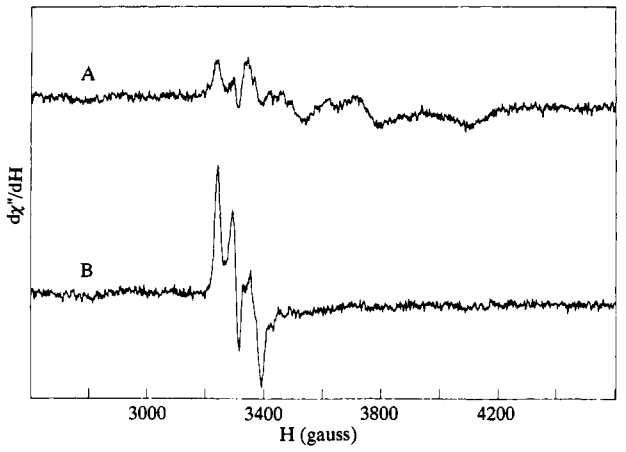

FIGURE 8: EPR spectra of CODH before (A) and after (B) oxidation of group II sites. (A) EPR spectrum of CODH prepared by adding just enough thionin to maximize A_{420} and momentarily turn the protein solution light blue in color. (B) EPR spectrum of CODH prepared by adding enough thionin to render the protein solution blue for over 4 min. The spectrometer conditions were as follows: microwave frequency, 9.419 GHz; modulation frequency, 100 kHz; modulation amplitude, 11.5 G; sweep time, 335 s; time constant, 0.163 s; microwave power, 0.05 mW; sample temperature, 10 K. The protein concentration was 27 μM. Spectra shown are the average of 10 scans. Signal intensities for the g_{av} = 1.82 and 2.03 signals in A and B, respectively, each corresponded to greater than 0.2 spin/mol, values comparable in intensity to other CODH signals.

of this site exhibited little change in A_{420} suggests either that it is not an iron–sulfur cluster or, if it is, that its optical and redox properties are quite unusual.

Summary

The many CODH redox reactions described in this paper can be categorized into three groups. Group I reactions arise from four redox sites, each capable of undergoing one-electron transfers. We have designated these sites $[Fe/S]_A$, $[Fe/S]_B$, A′, and B′. At least two of the group I sites are iron–sulfur clusters, including the $[Fe_4S_4]^{2+/1+}$ cluster in CODH. $[Fe/S]_B$ and B′ have reduction potentials below that needed to reduce protons (at pH 8), while $[Fe/S]_A$ and A′ have reduction potentials above that value. Either $[Fe/S]_B$ or B′, which ever is reduced last by CO, has a reduction potential near the CO/CO_2 couple (−622 mV). Group I redox reactions are likely to be involved in the catalytic mechanism of CODH.

Group II redox reactions arise from either one or two n = 1 sites, including the g_{av} = 1.82 species. These sites can be reduced by CO and oxidized by thionin. Their reduction potentials are more positive than those of group I, and they do not exhibit spectral changes at 420 nm when they change oxidation states. Hence, either these sites are not iron–sulfur clusters or they are iron–sulfur clusters with unusual optical and redox properties.

Group III oxidations occur when oxidants such as thionin are added in large molar amounts. These oxidations slowly inactivate CODH, with a rate that declines with increasing amounts of thionin. Exposure of 100 equiv/mol of thionin to CODH for 1 week causes complete inactivation. Group III reactions are not involved in the catalytic mechanism of the enzyme.

Added in Proof

Recent results suggest that the "tailing" near the ends of the CO reductive titrations (Figure 4) might be due to CO binding at the NiFeC complex rather than to the inability of CO to stoichiometrically reduce the site undergoing reduction. Experiments aimed at clarifying this are in progress.

Acknowledgments

We thank A. Ian Scott, Frank M. Raushel, and Jeffrey W. Kelly for the generous use of their equipment and Euiwha Moon for help with some of the preliminary studies.

Registry No. CODH, 64972-88-9; thionin, 581-64-6; CO, 630-08-0.

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Extensive Comparison of the Substrate Preferences of Two Subtilisins As Determined with Peptide Substrates Which Are Based on the Principle of Intramolecular Quenching†

Hanne Grøn, Morten Meldal, and Klaus Breddam*

Department of Chemistry, Carlsberg Laboratory, Gamle Carlsberg Vej 10, DK-2500 Copenhagen Valby, Denmark
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ABSTRACT: Subtilisins are serine endopeptidases with an extended binding cleft comprising at least eight binding subsites. Interestingly, subsites distant from the scissile bond play a dominant role in determining the specificity of the enzymes. The development of internally quenched fluorogenic substrates, which allow polypeptides of more than 11 amino acids to be inserted between the donor and the acceptor, has rendered it possible to perform a highly systematic mapping of the individual subsites of the active sites of subtilisin BPN′ from *Bacillus amyloliquefaciens* and Savinase from *Bacillus lentus*. For each enzyme, the eight positions S_5–S'_3 were characterized by determination of k_{cat}/K_M values for the hydrolysis of substrates in which the amino acids were systematically varied. The results emphasize that in both subtilisin BPN′ and Savinase interactions between substrate and S_4 and S_1 are very important. However, it is apparent that interactions between other subsites and the substrate exert a significant influence on the substrate preference. The results are rationalized on the basis of the structural data available for the two enzymes.

The active site of a proteolytic enzyme is occasionally divided into a catalytic site and a binding site. The catalytic site consists of the limited number of amino acid residues which are directly involved in the breakage of the peptide bond, e.g., by functioning in proton transfer or binding of the oxyanion of the tetrahedral intermediate. The binding site is composed of a fairly large number of amino acid residues which secure the proper alignment of the substrate prior to catalysis. In addition, binding energy is used to transform the substrate into the transition state (Jencks, 1975), and thus, it is not entirely meaningful to distinguish between catalytic site and binding site.

The binding site as defined by Schechter and Berger (1967) may be divided into a number of subsites, each by multiple interactions securing the binding of a single amino acid residue from the substrate. The properties of the amino acid residues which constitute a given binding subsite determine which amino acid residue(s) of the substrate may bind, and thus, they provide the basis of subsite specificity or preference in the case of less restrictive subsites. The nature of these interactions may be studied by site-directed mutagenesis and chemical modifications, but it is a prerequisite that the individual subsites are carefully mapped. Although X-ray crystallography and NMR studies can provide information about which amino acids are involved in substrate binding, the side-chain preference of each subsite is best determined by systematic variations of substrate structures. Such studies have been performed over the past 20 years (Svendsen, 1976), but they were restricted by the means to follow hydrolysis and the availability of substrates (Meldal & Breddam, 1991). The recent development of highly efficient donor/acceptor pairs for substrates based on intramolecular fluorescence quenching, allowing the use of long peptide substrates spanning the entire binding site (Meldal & Breddam, 1991); Matayoshi et al., 1990), represents a significant improvement in this context. We here describe the complete subsite mapping of two enzymes belonging to the subtilisins which are known to have extensive binding sites and, yet, are considered to be unspecific but with a well-defined substrate preference. The known three-dimensional structures of these enzymes form the basis for the interpretation of the results.

Materials and Methods

Materials. Sephadex CM and Sepharose CL 6B were from Pharmacia LKB Biotechnology, Sweden, Bio-Gel P-4 was from Bio-Rad, Richmond, CA, and bacitracin–Sepharose was prepared as previously described (Mortensen et al., 1989). Macrosorp SPR-250 was purchased from Sterling Organics.

Solvents were distilled at the appropriate pressure in a packed column of Raschig rings. DMF[1] was analyzed by

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