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Insertion of New Sequences into the Catalytic Domain of an Enzyme†

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ABSTRACT: Activities of enzymes can be modified by the replacement of active-site amino acids with residues that strengthen specific interactions with substrates or that alter the specificity. The scope for engineered enzymes would be broadened if additional, new sequences could be inserted into a catalytic domain. Properly designed, these sequences could encode new ligand binding sites, be intermediates in the construction of chimeric enzymes, or alter the internal flexibility and "breathing" modes of the active-site region. As a first step toward this objective, we inserted oligopeptides of up to 14 amino acids into various locations within an 82 amino acid region of the adenylate synthesis domain of *Escherichia coli* methionyl-tRNA synthetase. These sites include ones that are flanked by sequences that are conserved between the proteins from *E. coli* and the yeast *Saccharomyces cerevisiae* and those that are essential for activity and stability. We found that all of the insertional mutants are stable and some have catalytic parameters for adenylate synthesis that are comparable to those of the wild-type enzyme. Thus, such an approach may provide for a variety of novel applications.

Modification of enzyme activity to change substrate interactions or alter specificity has been achieved by site-directed mutagnesis of active-site amino acids. Broader applications for enzyme design include the insertion of new ligand binding sites, alteration of internal flexibility (Bone et al., 1989), and the construction of chimeras that combine desired traits of two or more enzymes. Such novel characteristics require the introduction of protein segments into enzyme domains. To explore new approaches to enzyme engineering, we chose to insert oligopeptides into a member of a major class of enzymes.

The aminoacyl-tRNA synthetases catalyze the aminoacylation of specific transfer RNAs. The two-step reaction involves condensation of amino acid with adenosine triphosphate (ATP) to produce an enzyme-bound aminoacyl adenylate intermediate and subsequent transfer of the amino acid to tRNA to give aminoacyl-tRNA (Schimmel, 1987):

$$\text{ATP} + \text{AA} \rightleftarrows \text{AA-AMP} + \text{PPi} \quad (1)$$

$$\text{AA-AMP} + \text{tRNA} \rightleftarrows \text{AA-tRNA} + \text{AMP} \quad (2)$$

Although they catalyze the same chemical reaction, the synthetases vary considerably in their primary sequences and gross structural features (for example, different subunit sizes and quaternary structures) (Schimmel, 1987). However, crystallographic analysis has determined that a domain containing a Rossmann nucleotide fold (Rossmann et al., 1975) is found in the amino-terminal portion of two synthetases: *Escherichia coli* methionyl-tRNA synthetase and *Bacillus stearothermophilus* tyrosyl-tRNA synthetase (Bhat et al., 1982; Risler et al., 1981; Zelwer et al., 1982; Blow & Brick, 1985; Brunie et al., 1987). Some structural similarity between parts of the nucleotide folds of these two enzymes has been noted. Amino acid and ATP are bound in this domain (Bhat et al., 1982; Risler et al., 1981; Zelwar et al., 1982; Blow & Brick, 1985; Brunie et al., 1987), which presumably is the site of amino acid activation. Sequence analysis and structural modeling suggest that a number of other synthetases share this motif (Schimmel, 1987; Starzyk et al., 1987).

The roughly 361 amino acid α/β structure that forms the nucleotide fold of *E. coli* methionyl-tRNA synthetase is depicted in Figure 1 (Barker et al., 1982; Dardel et al., 1984; Brunie et al., 1987). The exact disposition of methionine and ATP in this structure is not yet clarified (Brunie, et al., 1987). A segment designated "connective polypeptide 1" (CP1)

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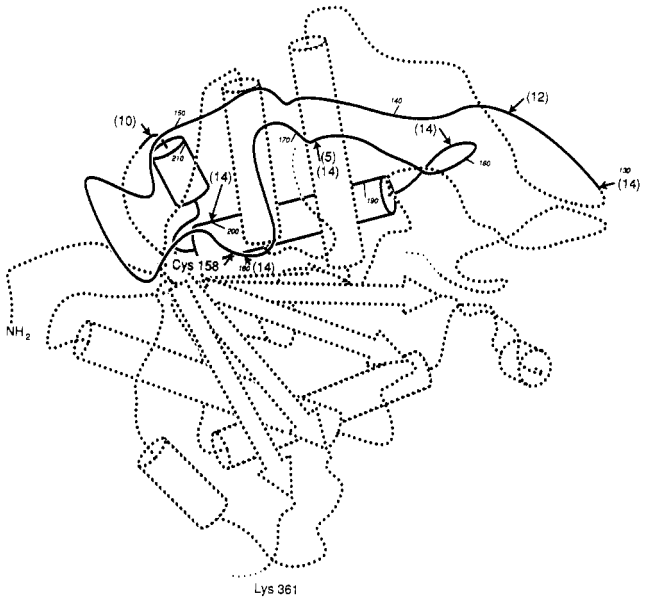

FIGURE 1: Three-dimensional representation of the nucleotide fold in *E. coli* methionyl-tRNA synthetase. Arrows indicate β-strands of the prototypical nucleotide fold, and cylinders indicate proposed α-helices. This representation is not exhaustive and is subject to refinement. The region of CP1 that was probed by site-directed insertional mutagenesis is colored in black. In this region every 10th amino acid is indicated, and arrows denote positions of insertions with their peptide lengths given in parentheses. This three-dimensional representation of the nucleotide fold of *E. coli* methionyl-tRNA synthetase is modeled after Color Plate 1 of Brunie et al. (1987) and Figure 1 of Starzyk et al. (1987).

(Starzyk et al., 1987) encompasses approximately amino acids 98–225 and, in the nomenclature used for lactate dehydrogenase (Rossmann et al., 1975), connects β_C to β_D. We obtained independent evidence that this segment is important for the adenylate synthesis activity and stability of the enzyme (see below) and subsequently chose to make a systematic investigation of the effects of insertions into different sites within this segment.

Materials and Methods

Insertional Mutagenesis. Site-directed insertional mutagenesis was carried out on plasmid pRS734.[1] This plasmid consists of a pUC19 (Yanisch-Perron et al., 1985) backbone with expression of the gene for methionyl-tRNA synthetase (Barker et al., 1982) under the control of the *lac* promoter and with the gene for the *lac* repressor (*lac i*; Calos et al., 1983) cloned into the *Eco*RI site. Upon induction with IPTG[2] a monomeric methionyl-tRNA synthetase truncated at Asp564 with a 17 amino acid C-terminal tail from pBR322 (Barker et al., 1982) is made. For the mutagenesis, the kanamycin resistance GenBlock gene cartridge (Pharmacia) was inserted at naturally occurring restriction sites and at additional sites created by site-directed mutagenesis [oligonucleotide-directed in vitro mutagenesis kit (Amersham)]. This results in random insertions, although in general the sequences of the inserted peptides favor an open Ω-loop structure. The following changes were made: Val160 changed to Ile to insert a *Bgl*II site; Ile171/Glu172 changed to Val/Asp to insert a *Sal*I site and an *Spe*I site; Met184 changed to Thr to insert a *Mlu*I site (this plasmid with the new *Mlu*I site does not encode the *lac i* gene); Met200 changed to Ile to insert a *Bgl*I site; Glu212/Gln213 changed to Asp/Leu to insert a *Bgl*II site; Cys158 changed to Ala. [All mutations of *metG* (the single gene that encodes methionyl-tRNA synthetase) that created new restriction sites encoded proteins with activities equal to that of the wild-type enzyme.] The GenBlock gene cartridge was then removed by digestion with the appropriate enzymes to produce insertions of 5–14 amino acids that were checked by DNA sequencing. During plasmid construction all transformations were made into strain DH1, a *rec*A$^-$ strain that is phenotypically wild type for methionyl-tRNA synthetase. Desirable plasmids then were used to transform tester strain MJ1000D, which is a methionine auxotroph that harbors a mutation in the gene for methionyl-tRNA synthetase (Barker et al., 1982).

Enzyme Purification and Kinetic Determinations. The wild-type methionyl-tRNA synthetase and P130 → PEFPGSVDRRIRGIP and M184 → TRIPRIRRSTDPGNS insertion proteins were synthesized upon addition of IPTG (1 mM) to the culture, and cells were subsequently disrupted by sonication in 50 mM potassium phosphate (pH 7.5), 25 mM dithiothreitol, 100 mM sodium chloride, and 500 μM phenylmethanesulfonyl fluoride (PMSF) Cell debris was removed by centrifugation, and the clarified supernatant was diluted in 25 mM Tris-HCl (pH 7.5), 1 mM dithiothreitol, and 500 μM PMSF and was applied to a Mono-Q HR 5/5 column (FPLC, Pharmacia) equilibrated in the same buffer. Methionyl-tRNA synthetase absorbs weakly to the Mono-Q column under these conditions and was eluted in the early fractions of a gradient made with 1 M NaCl in the starting buffer. The resulting preparation was estimated to be 50% pure. Enzyme concentration was determined by the adenylate burst assay (Fersht et al., 1975) using 1 mM methionine. The enzyme was assumed to be a monomer under the assay conditions, and activity was a linear function of protein concentration. Activity was measured as described below.

Enzyme Assays. Cell extracts (Toth & Schimmel, 1986) or purified column fractions (see above) were assayed for ATP–pyrophosphate exchange activity in triplicate at 37 °C (Calendar & Berg, 1966; Starzyk et al., 1982).[1] A constant amount of protein (determined by the Bio-Rad procedure) was added. All extracts were examined by gel electrophoresis prior to activity determinations, and the width of the band corresponding to the protein of interest was measured. This measurement then was used to estimate the amount of plasmid-encoded enzyme present in the extracts, and the resulting activities were normalized to these estimated enzyme levels.[1] Expression of wild-type methionyl-tRNA synthetase from pRS734 (see above) results in a 50-fold increase in activity relative to the activity of the single chromosomal copy in untransformed DH1. The relative activities of the insertion proteins were determined in the background of strain MJ1000D, which was transformed with the various insertion plasmids. This strain (MJ1000D) has a mutant methionyl-tRNA synthetase with an elevated K_M for methionine that is 350-fold above that of the wild-type enzyme (K_M = 2.5 mM under aminoacylation conditions; Barker et al., 1982), and the methionine-dependent ATP–PPi exchange activity is 10% that of DH1 extracts.

Results and Discussion

A sequence comparison between CP1 of the *E. coli* methionyl-tRNA synthetase and an analogous region in the *Saccharomyces cerevisiae* methionine enzyme (Walter et al., 1983) reveals a 36% identity for amino acids 98 to 191 of CP1 (Figure 2). This stretch includes four conserved cysteines. The identity drops to 15% for the remainder of CP1. Sub-

[1] Details are available upon request from R. Starzyk.

[2] Abbreviations: A, Ala; C, Cys; D, Asp; E, Glu; F, Phe; G, Gly; H, His; I, Ile; K, Lys; L, Leu; M, Met; N, Asn; P, Pro; Q, Gln; R, Arg; S, Ser; T, Thr; V, Val; W, Trp; Y, Tyr; IPTG, isopropyl β-D-thiogalactopyranoside.

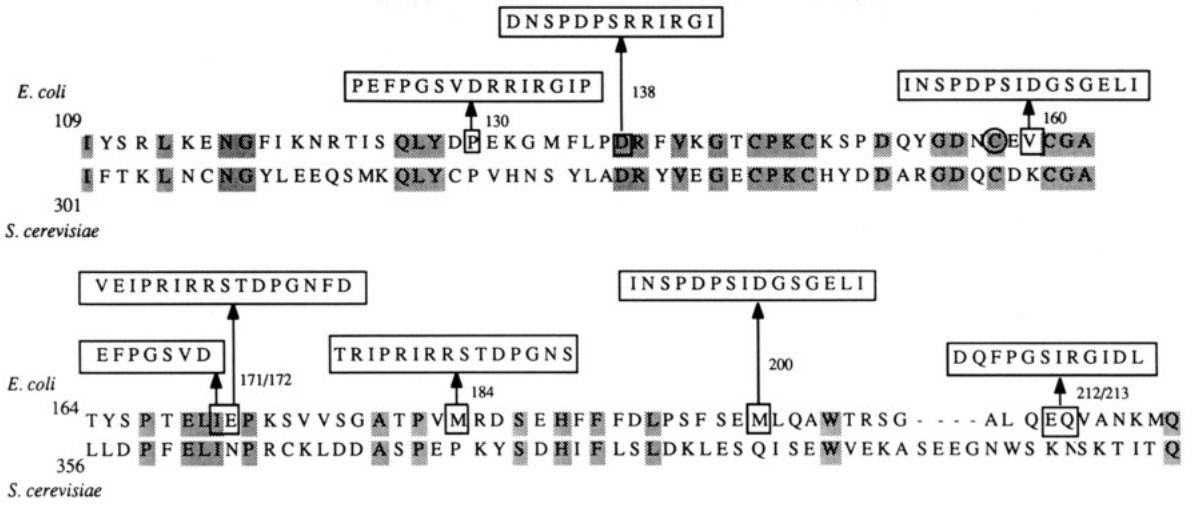

FIGURE 2: Locations and sequences of insertions in *E. coli* methionyl-tRNA synthetase. The top line shows the sequence of *E. coli* methionyl-tRNA synthetase from amino acid 109 to amino acid 219. The bottom line shows the analogous region from the methionine enzyme of *S. cerevisiae* aligned with the bacterial enzyme (Walter et al., 1983). Identical amino acids are shaded. In the bacterial sequence, the insertion sequences replace the boxed amino acid(s) as indicated.

stitution of alanine for the conserved C158[2] produces a stable but catalytically inactive enzyme (designated C158A, see below). In addition, deletion of L127–P137 produces a protein that is unstable in vivo (not shown). These results indicate that at least some amino acids in CP1 are essential for catalysis and structural stability.

We introduced new sequences into specific locations of CP1 that collectively include regions of higher and lower identity with the yeast enzyme (Figure 2). Site-directed insertional mutants were made in a plasmid that contains a *lac* promoter followed by a truncated version of *metG* (Barker et al., 1982) that ends at D564. This protein is slightly larger than the amino-terminal tryptic fragment whose crystal structure has been solved and which extends to K547 (Brunie et al., 1987). Random sequences ranging in size from 5 to 14 amino acids were inserted at eight locations between P130 and E212/Q213 (Figure 2). Many of the amino acids in the insertion sequences favor Ω-loop formation (Leszczynski & Rose, 1986). Upon addition of IPTG to the culture, a large amount of each of the eight "insertion" enzymes was made as well as of the C158A mutant protein (Figure 3). The level of methionyl-tRNA synthetase polypeptide in the cell lysates containing insertion and mutant enzymes is approximately within a factor of 2 of that of the plasmid-encoded wild-type enzyme (see Figure 4 and Materials and Methods). The insertion mutant proteins reproducibly migrate more slowly than the wild-type protein.

Enzyme activity in various extracts was measured by the methionine-dependent ATP–pyrophosphate exchange assay (Calendar & Berg, 1966; Starzyk et al., 1982) which monitors the activity that requires the nucleotide fold. These assays were performed with insertion and mutant plasmid constructs in strain MJ1000D (Barker et al., 1982). This strain bears a methionyl-tRNA synthetase that has a greatly elevated Michaelis constant (K_M) for methionine relative to that for the wild-type strain. Under the conditions of the assay, crude extracts of MJ1000D show very little methionine-dependent pyrophosphate exchange activity.

Figure 4 compares the activities of the following extracts: MJ1000D transformed with the wild-type plasmid and with various insertion and mutant plasmids, untransformed MJ1000D, and untransformed strain DH1 (which contains only the chromosomal copy of wild-type methionyl-tRNA synthetase). The activities are normalized to the amount of

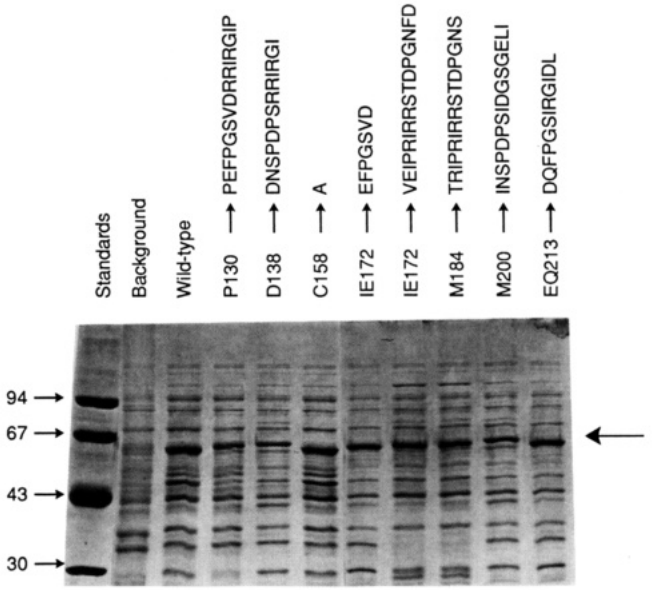

FIGURE 3: Visualization of stable insertion enzymes. The locations and sequences of the various insertions are indicated above their respective lanes and are as predicted from the gene sequence. Cell lysates of cultures induced with IPTG (1 mM) were analyzed on a Laemmli gel (Laemmli, 1970). Size markers (Pharmacia) are indicated on the left. Protein V160 → INSPDPSIDGSGELD is not shown on this gel, although it is easily detected in experiments analogous to those shown.

plasmid-borne methionyl-tRNA synthetase present in the crude extract; this value was estimated for the wild-type enzyme and for each insertion and mutant enzyme from the width of the appropriate bands on an SDS gel. The activity of a single wild-type chromosomal copy (from DH1) is taken to be 1.0, and the insertion enzyme activities are indicated relative to that value. We designated as "0" the activity (10% that of the DH1 level) of the single-copy MJ1000D allele that is present in all extracts containing enzymes with insertions. Values greater than 0 reflect subtraction of this background. Four of the eight insertion proteins have activities well above the amount contributed by the single wild-type *metG* allele of DH1. Of these four, two have activities identical with that of the amplified wild-type protein and the other two are approximately within a factor of 2. Additionally, three other insertion proteins have significant, albeit low, activities.

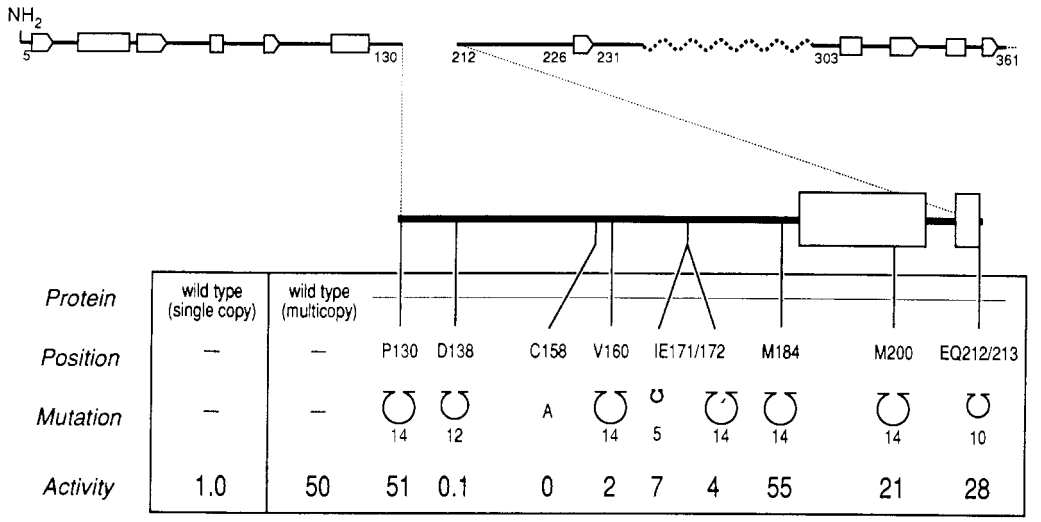

FIGURE 4: Relative activities of insertion enzymes. Secondary structural elements of the prototypical Rossmann fold of methionyl-tRNA synthetase are indicated as pentagons (β-strands) or rectangles (α-helices). The wavy line indicates the location of connective polypeptide 2, which connects β_D to α_E (Starzyk et al., 1987). This representation is modeled after Figure 6 of Starzyk et al. (1987). Enlarged is the region in CP1 between Pro130 and Glu212/Gln213, where all insertions were placed. To make an insetion, parent plasmid pRS734 was opened at a restriction site corresponding to one of the amino acid residues listed in the "position" row and, depending on the site, 5–14 codons were inserted in frame at the given site as indicated in the "mutation" row. (See Materials and Methods for details of the mutagenesis procedure.) The activity that is encoded by a single wild-type chromosomal copy of *metG*, the gene for methionyl-tRNA synthetase, is taken to be 1.0. (This activity was measured in protein extracts from strain DH1 which is phenotypically wild type for methionyl-tRNA synthetase.) The activities of the plasmid-encoded wild-type, insertion, and mutant enzymes are indicated relative to that value as listed in the "activity" row. Strain MJ1000D was the recipient strain for determinations of plasmid-encoded methionyl-tRNA synthetase activity (see text). See Materials and Methods for determinations of enzyme levels and activities.

Table I: Kinetic Parameters of Insertion Enzymes[a]

enzyme	K_M(Met) (μM)	K_M(ATP) (μM)	k_{cat} (s^{-1})	k_{cat}/K_M(Met) (10^6 M^{-1} s^{-1})	k_{cat}/K_M(ATP) (10^6 M^{-1} s^{-1})
wild type	38	295	83	2.2	0.28
M184 → TRIPRIRRSTDPGNS	61	194	23	0.38	0.12
P130 → PEFPGSVDRRIRGIP	86	633	94	1.1	0.15

[a] The values reported for k_{cat} were calculated from the known enzyme concentrations and from values of the apparent V_{max} ($V_{max,app}$) that were obtained from six Eadie–Hofstee plots (Eadie, 1942; Hofstee, 1959) (two for each enzyme) of $V_{max,app}$ versus $V_{max,app}$/[ATP] or versus $V_{max,app}$/[methionine]. The concentration range was 5–200 μM for methionine and 20–500 μM for ATP (at higher concentrations of ATP, the kinetics were not well-behaved).

Because the enzyme studied here is a monomer (Cassio & Waller, 1971; Barker et al., 1982), it is unlikely that the activities of enzymes with insertions result from association with the inactive MJ1000D synthetase. If this were the case, it is unlikely that the total activity generated would exceed that found in extracts of strain DH1. In contrast, we observe up to a 55-fold increase in methionine-dependent ATP–pyrophosphate exchange activity in extracts containing plasmid-encoded insertion enzymes. In addition, the presence in cell extracts of amplified mutant proteins of the expected size (Figure 3) argues against the possibility that activity is generated (in the active enzymes) through marker rescue by a genetic recombination which produces the full-length wild-type methionyl-tRNA synthetase. To further exclude this possibility, we performed assays in the presence of a different *metG* mutant allele (strain PL8-31, received from B. Bachmann) and obtained similar results.

We surmise that at least four (and possibly seven) of the eight gene constructions with insertions in CP1 encode proteins that are intrinsically active. Insertions generating the lowest activities cluster around C158 in the primary sequence (Figure 4) but appear to be well separated in the three-dimensional structure (Figure 1). In the CP1 segment of *E. coli* methionyl-tRNA synthetase, the sequence around C158 is the most highly conserved with the analogous region of the yeast enzyme.

We determined the kinetic parameters K_M (for ATP and methionine) and k_{cat} in the ATP–pyrophosphate exchange assay for the partially purified wild-type enzyme and for two partially purified mutant enzymes: P130 → PEFPGSVDRRIRGIP and M184 → TRIPRIRRSTDPGNS. Enzyme concentrations were determined by active-site titrations (Fersht et al., 1975). The three parameters for each of the two insertion enzymes are within 2–4-fold of the respective values for the wild-type enzyme (Table I). In the case of insertion enzyme P130 → PEFPGSVDRRIRGIP, the k_{cat} is similar to that for the wild-type synthetase. With insertion enzyme M184 → TRIPRIRRSTDPGNS, the K_M for ATP is even lower than that of the wild-type protein. Thus, at these two locations in the catalytic structure insertions of 14 amino acids are integrated with little perturbation of substrate kinetic parameters.

Structural analysis of these insertion proteins would add to our understanding of structure–function relationships in enzymes with Rossmann folds in general and in aminoacyl-tRNA synthetases in particular. Each of the insertion proteins studied here is sufficiently stable to detect in cell extracts (Figure 3). It is noteworthy that the M200 → INSPDPSIDGSGELI and the E212/Q213 → DQFPGSIRGIDL insertions are at the C-terminal ends of two proposed helices (Brunie et al., 1987) (see Figures 1 and 2) and yield active enzymes (Figure 4). In the three-dimensional representation of methionyl-tRNA

synthetase, M200 is close to C158 and also to V160, where a V160 → INSPDPSIDGSGELI insertion produces a marginally active enzyme. Additionally, the site of insertion for the active P130 → PEFPGSVDRRIRGIP protein is only eight residues from the completely inactive D138 → DNSPDPSRRIRGI insertion protein; both of these insertions are into a loop near the surface of the enzyme (Figure 1). A more detailed interpretation of these results awaits publication of the three-dimensional structural coordinates of the enzyme.

Earlier, we proposed that, like the *E. coli* methionine enzyme, *E. coli* isoleucyl-tRNA synthetase has a Rossmann fold in its amino-terminal portion with a CP1 segment that is 3 times the length (approximately 320 amino acids) of the CP1 segment of methionyl-tRNA synthetase (Starzyk et al., 1987). Sequences that collectively span 145 amino acids (or nearly half) can be deleted from CP1 of isoleucyl tRNA synthetase without eliminating catalytic activity (Starzyk et al., 1987). So far there is no evidence that any sequence in the much smaller CP1 of methionyl-tRNA synthetase can be similarly deleted. Because there is no sequence similarity between the regions that correspond to CP1 in the two enzymes, and because no crystal structure is available for the isoleucine enzyme, it is not yet possible to determine the relationship of the locations of the "active" insertions in the methionine enzyme to the dispensable sequences in isoleucyl-tRNA synthetase.

In this work we have introduced new sequences into the catalytic domain of an aminoacyl-tRNA synthetase to test the potential of insertion mutagenesis for enzyme engineering. The highly active insertions P130 → PEFPGSVDRRIRGIP and M184 → TRIPRIRRSTDPGNS were made into relatively well conserved regions so that sequence conservation is not a reliable indication of whether a given insertion will produce an active enzyme. Furthermore, every insertion we attempted resulted in a stable protein. This suggests that the overall structural stability of the core protein is not especially sensitive to the addition of peripheral peptides. Although the peptides we inserted favor an open, Ω-loop structure (Leszczynski & Rose, 1986), the absence of tightly packed secondary structural elements may not be a strict requirement for an insertion peptide.

While saturation mutagenesis has contributed precise information on the role of individual residues in a small part of a well-defined protein (Reidhaar-Olson & Sauer, 1988), codon insertion mutagenesis has been used to probe protein structure on a broader scale to map functional domains in sensitive structural regions of a protein of unknown structure. A conclusion from the codon insertion studies (Boeke, 1981; Stone et al., 1984; Barany, 1985a,b; Freimuth & Ginsberg, 1986; Barany, 1987) is that smaller insertions (such as two codons) do not diminish catalytic activity as much as larger ones. In the present work with a protein whose structure is known in some detail, we observe that, depending on the location, up to 14 codons can be inserted without loss of enzyme activity. While we did not investigate 2 or 4 codon insertions at the sensitive positions D138 and V160, we did check the effects of a 5- versus a 14-codon insertion at IE 171/172 but observed only a 2-fold decrease in the marginal level of activity with the longer insertion (which, however, also has a different sequence) (Figures 2 and 4).

It is noteworthy that 7 of 10 consecutive amino acids in the P130 insertion are identical with a sequence that forms the end of one and the beginning of another α-helix in and adjacent to the glycogen storage site of glycogen phosphorylase *b* (Sansom et al., 1985). Having now defined sites where insertions can be placed in methionyl-tRNA synthetase, it will be of interest to test sequences with various characteristics. In future applications, a ligand binding site may be introduced that could make methionyl-tRNA synthetase sensitive to the new ligand, or a sequence element could be added which would facilitate association of the methionine enzyme with another protein. The sites of the active insertions might also represent places where linkages could be made for the construction of chimeric aminoacyl-tRNA synthetases. It also will be of interest to explore these concepts and applications with other enzymes.

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Oxidation–Reduction Potentials and Ionization States of Extracellular Peroxidases from the Lignin-Degrading Fungus *Phanerochaete chrysosporium*†

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ABSTRACT: The oxidation–reduction potentials of lignin peroxidase isozymes H1, H2, H8, and H10 as well as the Mn-dependent peroxidase isozymes H3 and H4 are reported. The potentiometric titrations involving the ferrous and ferric states of the enzyme had Nernst plots indicating single-electron transfer. The E_{m7} values of lignin peroxidase isozymes H1, H2, H8, and H10 are −142, −135, −137, and −127 mV versus standard hydrogen electrode, respectively. The E_{m7} values for the Mn-dependent peroxidase isozymes H3 and H4 are −88 and −93 mV versus standard hydrogen electrode, respectively. The midpoint potential of H1, H8, and H4 remained unchanged in the presence of their respective substrates, veratryl alcohol and Mn(II). The midpoint potential between the ferric and ferrous forms of isozymes H1 and H4 exhibited a pH-dependent change between pH 3.5 and pH 6.5. These results indicate that the reductive half-reaction of the enzymes is the following: ferric peroxidase + $1e^-$ + H^+ → ferrous peroxidase. Above pH 6.5, the effect of pH on the midpoint potential is diminished and indicates that an ionization with an apparent pK_a equal to approximately 6.6–6.7 occurs in the reduced form of the enzymes. A heme-linked ionization group in the ferrous form of the enzymes was confirmed by studying the effect of pH on the absorption spectra of isozymes H1 and H4. These spectrophotometric pH titration experiments confirmed the electrochemical results indicating pK_a values of 6.59 and 6.69 for reduced isozymes H1 and H4, respectively. These results indicate the presence of a heme-linked ionization of an amino acid in the reduced form of the lignin peroxidase isozymes similar to that of other plant peroxidases.

The oxidation–reduction potentials of the ferric/ferrous couple of the lignin peroxidase (ligninase) isozymes and the Mn-dependent peroxidase isozymes of *Phanerochaete chrysosporium* were determined in the present study. These two classes of isozymes are the predominant enzymes produced by the white-rot fungus *P. chrysosporium* and are thought to impart this wood-destroying fungus the ability to degrade lignin (Tien & Kirk, 1984; Glenn & Gold, 1985; Paszczynski et al., 1986). Both types of isozymes are heme-containing glycoprotein peroxidases. The heme iron in the native protein is in the high-spin, pentacoordinate ferric state with a histidine residue coordinated as the fifth ligand (Tien & Kirk, 1984; Kuila et al., 1985; Andersson et al., 1987). The primary structures deduced from sequencing the cDNA encoding for the lignin peroxidases (Tien & Tu, 1987a,b; de Boer et al., 1987) and Mn-dependent peroxidases (Pribnow et al., 1989; Pease et al., 1989) reveal homology of active-site residues with other peroxidases.

The lignin and the Mn-dependent peroxidases show different substrate specificity. The lignin peroxidase isozymes oxidize not only lignin and lignin model compounds (Tien & Kirk, 1983; Glenn et al., 1983; Higuchi, 1985) but also a large number of other organic compounds (Kamaya & Higuchi, 1984; Kersten et al., 1985; Hammel et al., 1986; Kirk et al., 1986). The Mn-dependent peroxidase isozymes oxidize a number of phenolic compounds and dyes as well as several amines (Kuwahara et al., 1984; Glenn & Gold, 1985; Paszczynski et al., 1986). Like other peroxidases, both the lignin and Mn-dependent peroxidases utilize H_2O_2 as the oxidant to achieve the higher oxidation state of compound I, which is 2 oxidation equivalents above the native enzyme (Chance, 1952). Compound I returns to native enzyme by sequential one-electron oxidations of aromatic substrates by way of the one-electron oxidized intermediate compound II (Nakamura et al., 1985; Andrawis et al., 1988). Lignin peroxidases utilize aromatic substrates whereas the Mn-de-

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