Identification of Structurally and Functionally Important Histidine Residues in Cytoplasmic Aspartyl-tRNA Synthetase from *Saccharomyces cerevisiae*†

S. Gasparini, P. Vincendon, G. Eriani, J. Gangloff, Y. Boulanger,* J. Reinbolt, and D. Kern

Laboratoire de Biochimie, Institut de Biologie Moléculaire et Cellulaire du Centre National de la Recherche Scientifique, 15, rue René Descartes, F-67084 Strasbourg Cedex, France

Received November 2, 1990; Revised Manuscript Received January 23, 1991

ABSTRACT: Cytoplasmic aspartyl-tRNA synthetase from *Saccharomyces cerevisiae* is an α_2 dimer (α, M_r 63 000), each α containing 12 histidines. The covalent incorporation of 6–7 mol of diethyl pyrocarbonate per monomer corresponded to complete enzyme inactivation. This inactivation was reversed by hydroxylamine hydrolysis which regenerates free histidine (and tyrosine) while leaving the carbethoxy group still attached to the ϵ-amino group of lysine. Three histidines, one tyrosine, and four lysines were the main targets of the reagent. Site-directed mutagenesis was also tried to replace each of these modified residues. Given the unstability of the carbethoxy–imidazole bond, the nine histidines that were not modified by diethyl pyrocarbonate were mutated too. For these experiments, the enzyme was expressed in *Escherichia coli* by using a vector bearing the structural gene in which the first 13 codons were replaced by the first 14 of the CII λ gene. This substitution had no effect on the kinetic parameters. The combined results of chemical modification and site-directed mutagenesis show that one histidine seems to be part of the active site while two others play an important structural role. On the other hand, labeled lysines and tyrosine are nonessential residues. These results are discussed in light of two recent articles establishing the existence of a second family of aminoacyl-tRNA synthetases devoid of the HIGH and KMSKS consensus sequences and containing no Rossmann's domain in their three-dimensional structures.

As recently published, cytoplasmic aspartyl-tRNA synthetase from *Saccharomyces cerevisiae* is a dimer made up of identical subunits (M_r 63 000) each containing three cysteine residues that are not involved in either substrate binding or catalysis (Kern et al., 1990). This was established by using both chemical modification and site-directed mutagenesis. Each enzyme monomer also contains 12 histidine residues, and it was previously shown that their chemical modification by diethyl pyrocarbonate (DEPC)[1] was accompanied by a sharp decrease in the rates of both amino acid activation and tRNA aminoacylation (Kern, unpublished results). Such a result goes along with many studies carried out over the past decade which all point at histidines as structurally or functionally important residues in the family of aminoacyl-tRNA synthetases.

Indeed, Barker and Winter first identified a conserved HI(L)GH sequence between tyrosyl-tRNA synthetase from *Bacillus stearothermophilus* and methionyl-tRNA synthetase from *Escherichia coli* in the N-terminal section of these two bacterial enzymes (Barker & Winter, 1982), and this sequence was later found in seven other enzymes from various sources as reported in a recent review (Burbaum et al., 1990). It was then proposed that this conserved stretch was a sort of signature sequence indicative of the nucleotide binding domain or Rossmann's fold found in the three-dimensional structures of the two above bacterial enzymes (Rossmann et al., 1975; Zelwer et al., 1982; Blow et al., 1983; Webster et al., 1987). This first group of nine synthetases, glutamyl-, glutaminyl-, arginyl-, tyrosyl-, methionyl-, tryptophanyl-, valyl-, isoleucyl-, and leucyl-tRNA synthetases, referred to as class I, also shares a common motif, KMSKS, which was shown to be close to the binding site for the 3′ end of tRNA (Hountondji et al., 1986). Site-directed mutagenesis further emphasized the importance of the HIGH(N) conserved signature sequence, and in the case of tyrosyl-tRNA synthetase, it was established that its two histidines (residues 45 and 48) were involved in ATP binding (Carter et al., 1984; Fersht et al., 1985; Lowe et al., 1985; Leatherbarrow et al., 1985; Jones et al., 1986).

Unfortunately, aspartyl-tRNA synthetase from *Saccharomyces cerevisiae* belongs to another group of synthetases that do not contain either of the two conserved motifs and besides its three-dimensional structure is not available at high resolution yet. This is the reason why a strategy similar to that described for cysteine residues was adopted. By combining the results of chemical modification by DEPC with those of site-directed mutagenesis, we could identify some histidine residues which play an important part in the enzyme activity. These results bring some further support to the idea of a second class of aminoacyl-tRNA synthetases which contain neither the HIGH nor the KMSKS consensus sequences as recently proposed (Eriani et al., 1990; Cusack et al., 1990).

Experimental Procedures

General. L-[^{14}C]Aspartic acid (200 mCi/mmol) and [^{14}C]ATP (400 mCi/mmol) were from the Commissariat â l'Energie Atomique (Saclay, France). [^{14}C]Diethyl pyrocarbonate[1] (7.3 mCi/mmol) was from Sigma (St. Louis, MO). [α-^{35}S]dATP (400 mCi/mmol) and ^{125}I-labeled protein A (34 mCi/mg) were from Amersham (U.K.).

Nonlabeled 2′-deoxynucleoside triphosphates and 2′,3′-dideoxynucleoside triphosphates were from Pharmacia (Uppsala, Sweden), nitrocellulose filters (0.22 or 0.45 μm) were from Millipore (Molsheim, France), and chymotrypsin was from Worthington (Freehold, NJ). Total brewers' yeast tRNA was from Boehringer (Mannheim, FRG).

† This work was supported by grants from the Centre National de la Recherche Scientifique.

* To whom correspondence should be addressed.

[1] Abbreviations: DEPC, diethyl pyrocarbonate; PTH, phenylthiohydantoin; HPLC, high-performance liquid chromatography; chymotrypsin, EC 3.4.21.1; T_4 DNA ligase, EC 6.5.1.1; T_7 DNA polymerase, EC 2.7.7.7.

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All chemicals were from Merck (Darmstadt, FRG), analytical grade, and so was the reverse-phase C18 column (Lichrocart 12.5 × 0.4 cm, particle size 5 μm).

Microconcentration Centricon cells and YM30 membranes were from Amicon (Epernon, France). Whatman 3MM paper disks were from Whatman (Maidstone, U.K.).

Enzymes[1] *and tRNA.* Aspartyl-tRNA synthetase (EC 6.1.1.12) from bakers' yeast was purified as described (Lorber et al., 1983). The specific activity measured in standard amino acid mixtures was 600–800 units/mg of protein; the molar enzyme concentration was determined from the absorbance (A_{280} = 0.60 mg mL^{-1} cm^{-1}) with a molecular weight of 125 000 (Kern et al., 1985). Brewers' yeast $tRNA^{Asp}$ was prepared according to previously published procedures (Keith et al., 1971; Dock et al., 1984); its acceptance capacity was 1600 pmol/A_{260} unit. Restriction endonucleases and T_4 DNA ligase were from Boehringer (Mannheim, FRG), and T_7 DNA polymerase was from Pharmacia (Uppsala, Sweden).

Activity Measurements. For both the ATP–PP_i exchange and tRNA aminoacylation reactions, the conditions and reagents were those reported (Kern et al., 1985).

Labeling Experiments. A typical reaction mixture contained aspartyl-tRNA synthetase (2.7 μM), glycerol (10% by volume), and various concentrations of [^{14}C]DEPC (from 0.3 to 1.2 mM) in 100 mM potassium phosphate buffer, pH 6 or 8. The reagent was stored in acetonitrile and diluted in the mixture to reach the desired concentration [with acetonitrile always lower then 2% (v/v) in order not to affect the enzyme activity]. The mixture was incubated at 4 °C. At different time intervals, aliquots were withdrawn and either assayed for both the ATP–PP_i exchange and tRNA aminacylation reactions (Kern et al., 1985) or precipitated onto Whatman 3MM paper disks in the presence of trichloroacetic acid (5% w/v). The disks were washed 3 times by an aqueous solution of trichloroacetic acid (5% w/v), rinsed with ethanol, dried, and subjected to scintillation counting as described (Kern et al., 1985).

Aliquots were also treated overnight at room temperature by 0.1 M hydroxylamine, pH 7 (to reverse histidine modification), and treated as above to measure the nonreversible incorporation of DEPC into the protein.

After 2 h of incubation at 4 °C, the reaction mixture was dialyzed against 0.1 M *N*-methylmorpholine acetate buffer, pH 8, and concentrated by using Centricon Amicon cells equipped with a YM30 membrane to reach a final protein concentration of 1–3 mg/mL.

Enzymatic Digestion of the Modified Enzyme and Isolation and Sequencing of the Labeled Peptides. After digestion of the modified aspartyl-tRNA synthetase with chymotrypsin for 4 h (with a protease:protein ratio of 5% w/w), the labeled peptides were purified by using HPLC on a reverse-phase C18 column as described in previous works (Hounwanou et al., 1983; Reinbolt et al., 1983; Kern et al., 1990). These peptides were sequenced by using an Applied Biosystems 470A gas-phase sequencer (equipped with a PTH 120A analyzer).

Bacterial Strains, Plasmids, Growth Media, and Transformation. The *E. coli* K12 TG1 strain used for site-directed mutagenesis was provided with the Amersham kit. The replicative form and single-stranded DNA of M13 were obtained from the *E. coli* JM 103 strain. Aspartyl-tRNA synthetase was expressed in the *E. coli* TGE 900 strain after its structural gene (APS) was cloned into the expression vector pTG 908 in the unique *Bam*HI restriction site: in this construction, the first 13 codons of aspartyl-tRNA synthetase were replaced by the first 14 of the CII λ gene so that the enzyme was expressed at a high level under the control of the CII λ promotor (Courtney et al., 1984; Tessier et al., 1986). Details concerning this construction will be published elsewhere (J. Gangloff, personal communication). For site-directed mutagenesis, the *Bam*HI–*Bam*HI fragment bearing the structural gene (APS) was subcloned into the replicative form of M13 mp18. Strains were grown in 1% tryptone, 0.5% yeast extract, 0.5% NaCl (w/v), and 1 mM NaOH with or without 2% gelose (w/v) at 37 °C. *E. coli* transformations and large-scale plasmid and phage DNA isolation were carried out as described (Maniatis et al., 1982).

Oligonucleotide Synthesis, Site-Directed Mutagenesis, and DNA Sequencing. Oligonucleotides (20–30 bases long) were complementary to the single-stranded DNA except for the replacement of the histidine codon (CAC) by those of glycine (GGC), alanine (GCT), or glutamine (CAA). An Applied Biosystems 318 A synthesizer was used for automated synthesis, and oligonucleotides were purified by HPLC on a reverse-phase C18 column. Site-directed mutagenesis was performed by means of the Amersham kit according to the procedures described (Nakamaye & Eckstein, 1988; Sayers et al., 1988a,b). The mutated DNA was identified by sequencing using the chain termination method (Tabor & Richardson, 1987).

Preparation of Crude Extracts, Enzyme Titration, and Determination of Its Kinetic Parameters. Clones were grown overnight at 37 °C in the above medium. After centrifugation, cells were suspended in 1 mL of 250 mM Tris-HCl buffer, pH 8, containing 1 mM $MgCl_2$, 10^{-4} M EDTA, 10^{-4} M dithioerythritol, 5 mM 2-mercaptoethanol, and 10^{-4} M diisopropyl fluorophosphate. The suspension was sonicated for periods of 20 s (total time 3 min) in an Ultrasons-Annemasse sonicator. Cell lysates were centrifuged and dialyzed overnight against a 50 mM potassium phosphate buffer, pH 7.2, containing the same additives as above plus 10% glycerol (v/v) (for temporary storage at −20 °C, a concentration of 50% glycerol was used). Protein concentration was estimated as published (Ehresmann et al., 1979), immunological titration of aspartyl-tRNA synthetase and determination of its kinetic parameters were performed according to Prévost et al. (1989). Western blotting analyses were also used to estimate the amount of mutated aspartyl-tRNA synthetase (Towbin et al., 1979).

Results

DEPC Modification and Enzyme Inactivation. Three different concentrations of the reagent were tried at pH 8 (0.3, 1, and 1.2 mM) versus four at pH 6 (0.75, 1, 2, and 3 mM) in order to select the best labeling conditions under which a maximum yield of inactivation could be reached for a minimum number of DEPC molecules incorporated per enzyme monomer. This was found at pH 6 where a plateau value of 6 mol of DEPC per monomer was reached after 2 h of incubation at 4 °C (whatever the reagent concentration) with a residual aminoacylation activity amounting to 10% of the initial one (Figure 1a). The inactivation was stoichiometric (Figure 1b) with an extrapolated value of 6–7 mol of DEPC per subunit for total inactivation. After hydroxylamine treatment, only 2 mol of DEPC per subunit was found, and a 50% recovery of the initial aminoacylation activity was measured. This 50% recovery of activity was observed too when a nonlabeled enzyme was treated with hydroxylamine under identical conditions. This suggests that only those residues reversibly modified by DEPC (histidine and/or tyrosine) could be important for enzyme activity. Indeed, their modification (as followed by absorbance measurements at 242

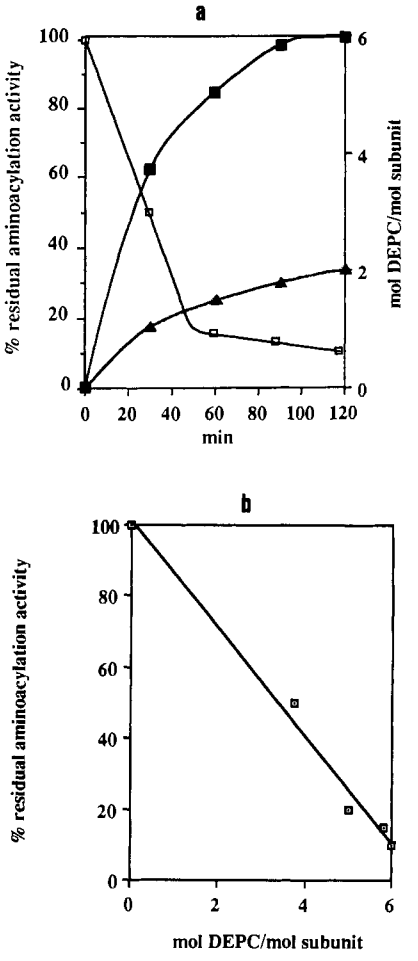

FIGURE 1: Inactivation of aspartyl-tRNA synthetase by DEPC at pH 6. (a) (■) Covalent incorporation of DEPC expressed as number of moles of radioactive DEPC per mole of subunit; (▲), number of moles of radioactive DEPC per mole of subunit after hydroxylamine hydrolysis; (□), residual aminoacylation activity expressed as a percentage of the initial one. (b) (⊡) Percent residual aminoacylation activity versus number of moles of radioactive DEPC per mole of subunit.

nm which do not detect lysine labeling) was of the first-order type, and the corresponding rate constant (0.15 min^{-1}) was close to those of inactivation for both ATP–PP_i exchange and aminoacylation reactions (respectively 0.10 and 0.11 min^{-1}). Total inactivation corresponded to an extrapolated value of 4 mol of DEPC per monomer. Therefore, these reversibly labeled residues seem to have the same reactivity. At pH 8, the same yield of inactivation was reached after 2 h at 4 °C, and up to 15 DEPC molecules were incorporated into each enzyme subunit, of which 6 were not removed by hydroxylamine hydrolysis.

In view of these findings, the following conditions were chosen for labeled peptides isolation: aspartyl-tRNA synthetase (2.7 μM) was incubated at pH 6 for 2 h at 4 °C in the presence of 1 mM radioactive DEPC. Under such conditions, a residual activity of 10% was measured for both the ATP–PP_i exchange and tRNA aminoacylation reactions.

Labeled Peptide Isolation and Peptide Sequencing. Given the fact that DEPC modification of the imidazole ring of histidine is rather unstable as compared to that of the ε-amino group of lysine (Melchior & Fahrney, 1970), a short chymotryptic digestion was carried out (4 h), and HPLC on a reverse-phase column was used for rapid purification of the labeled peptides. Control experiments performed on a synthetic GHK peptide showed that under the conditions selected

Table I: Sequences of the Major Radioactive Peptides Labeled by DEPC

peptide	position in sequence	modified residue[a]	amount sequenced (pmol)[b]
GKLPL	76–80	K77	20
GLVKAN	139–144	K142	30
VKANKEGTISKN	141–152	K142	20
VKANKEGTISKN	141–152	K142 and K151	30
EIHITKIY	188–195	H190	20
KIY	193–195	Y195	80
RHMTEF	333–338	H334	25
AHEIEL	374–379	H375	95
VRKQYPVEEF	380–389	K382	40
KQYP	382–385	K382	10
KQYPVEEF	382–389	K382	20

[a] Modified Lys and His were identified as their PTH derivatives at the corresponding sequencing step: N^{ϵ}-carbethoxy-α-PTH–Lys and carbethoxyimidazole-α-PTH–His, respectively. As for Tyr, no modified PTH derivative was found on sequencing of peptide KIY, indicating that the label was lost, most probably during incubation in trifluoroacetic acid. Therefore, the presence of modified Tyr-195 was deduced from the fact that KIY was the unique peptide found in the corresponding radioactive peak eluted from the reverse-phase column. [b] There was always a good correlation between the amount of peptide sequenced and that calculated in the radioactive peak from radioactivity measurements.

both lysine and histidine side chains were modified and that modification of the latter survived the isolation procedure. Free lysine and histidine were also treated with DEPC in order to identify the positions of the PTH derivatives of these two modified amino acids on the chromatograms delivered by the sequencer. Two independent sets of labeling experiments were performed on two different samples of aspartyl-tRNA synthetase, and the results of major radioactive peptide purification and sequencing are listed in Table I. As already emphasized (Kern et al., 1990), some of the radioactive peaks eluted from the reverse-phase column used to fractionate the chymotryptic digest contained peptide mixtures (even after a second chromatographic step), but such a mixture could often be solved unambiguously after microsequencing, with the support of the enzyme primary structure. Altogether, three histidines (190, 334, and 375), one tyrosine (195), and four lysines (77, 142, 151, and 382) appear to be the main targets of the chemical reagent. It is worth stressing that at least two of these four lysines were partially labeled, namely, 142 and 151, since the corresponding chymotryptic peptide (141–152) was present in three different peaks eluted from the reverse-phase column: one corresponded to the native peptide with no modified lysines, a second one to the peptide in which Lys-142 was labeled, and a third one to the peptide with both Lys-142 and -151 modified. Similarly, the isolation of radioactive peptides EI*H*ITKIY and KI*Y* (with modified His-190 and Tyr-195, respectively, Table I) shows that Tyr-195 was partially labeled. This means that the above plateau value of 6 mol of DEPC incorporated into each monomer reflects some statistical labeling of more than six well-defined targets. As shown in Figure 2, these main labeled amino acids are rather evenly distributed along the protein sequence.

Site-Directed Mutagenesis. As indicated under Experimental Procedures, the enzyme used for this work had its first 13 amino acids replaced by the first 14 or CII λ, and it was checked that this substitution had no effect at all on its kinetic parameters (Kern et al., 1990).

In the first series of experiments, each of the 12 histidines contained in each subunit was substituted for glycine in order to test the effect of complete side chain removal. For eight of these residues, namely, His-190, -350, -352, -375, -487, -489,

Table II: Substitution of Histidine Residues[a]

position of histidine	AA introduced	rate of tRNA aminoacylation (% of control)	rate of ATP–PP_i exchange (% of control)	K_m (μM) for Asp	K_m (μM) for ATP
control (overproducing strain)	no mutations	100	100	700	90
His-116	Gly	100	65	490	60
His-271	Gly	0	0		
	Ala	75	80–150	1100	135
	Gln	80	100	690	200
His-332	Gly	30–50	100	750	500–600
	Ala	100	125	1100	300
	Gln	100	140	1200	310
His-334	Gly	0.1	29	350	580
	Ala	3	35	300	700
	Gln	100	10	4200	1000
	Arg	25	100	50	120

[a] The activities were determined at substrate concentrations close to saturation levels. They were expressed as percentages relative to the values measured for the overproducing strain [12 000 and 3600 nmol of product (mg of enzyme)$^{-1}$ min^{-1} for the ATP–PP_i exchange and tRNA aminoacylation reactions, respectively]. Since the measurements were made in crude extracts of *E. coli*, it must be stressed that the bacterial aspartyl-tRNA synthetase does not aminoacylate $tRNA^{Asp}$ from yeast. On the other hand, the eukaryotic enzyme weakly charges the bacterial $tRNA^{Asp}$. This is not a problem since samples were diluted prior to the test, which makes the amount of *E. coli* $tRNA^{Asp}$ negligible with respect to the yeast species present in the aminoacylation mixture.

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  1  MSQDENIVKA VEESAEPAQV ILGEDGKPLS KKALKKLQKE QEKQRKKEER
 51  ALQLEAEREA REKKAAAEDT AKDNYGKLPL IQSRDSDRTG QKRVKFVDLD
101  EAKDSDKEVL FRARVHNTRQ QGATLAFLTL RQQASLIQGL VKANKEGTIS
151  KNMVKWAGSL NLESIVLVRG IVKKVDEPIK SATVQNLEIH ITKIYTISET
201  PEALPILLED ASRSEAEAEA AGLPVVNLDT RLDYRVIDLR TVTNQAIFRI
251  QAGVCELFRE YLATKKFTEV HTPKLLGAPS EGGSSVFEVT YFKGKAYLAQ
301  SPQFNKQQLI VADFERVYEI GPVFRAENSN THRHMTEFTG LDMEMAFEEH
351  YHEVLDTLSE LFVFIFSELP KRFAHEIELV RKQYPVEEFK LPKDGKMVRL
401  TYKEGIEMLR AAGKEIGDFE DLSTENEKFL GKLVRDKYDT DFYILDKFPL
451  EIRPFYTMPD PANPKYSNSY DFFMRGEEIL SGAQRIHDHA LLQERMKAHG
501  LSPEDPGLKD YCDGFSYGCP PHAGGGIGLE RVVMFYLDLK NIRRASFVPR
551  DPKRLRP*
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FIGURE 2: Distribution of the labeled residues along the amino acid sequence. (▼) Labeled histidine; (▽) labeled tyrosine; (●) labeled lysine.

-499, and -522, this mutation had no detectable effect on either the K_m values for the small substrates (L-Asp and ATP) or the rates of the ATP–PP_i exchange and tRNA aminoacylation reactions (data not shown).

Obviously, these eight residues are not involved in substrate binding and/or catalysis, and they play little if any part in maintaining a native enzyme three-dimensional structure. It is worth mentioning that two of these eight residues (190 and 375) were among the major targets of DEPC modification and this shows that not all the chemically labeled residues are essential for the enzyme activities (amino acid activation and tRNA aminoacylation). The results of the other four substitutions are listed in Table II: substituting His-116 for Gly slightly reduced the rate of amino acid activation without affecting the other kinetic parameters (K_m for the small ligands and overall rate of tRNA aspartylation). The mutation His-271 → Gly completely destroyed both enzyme activities while that of His-332 yielded a 60% decrease in the rate of tRNA aminoacylation with no significant changes in the other parameters (except perhaps for a 4–5-fold increase in the K_m for ATP). Finally, the last mutation, His-334 → Gly, yielded an enzyme for which both rates were reduced (70% decrease for amino acid activation and very close to a 100% decrease for tRNA aspartylation) while the K_m for ATP was slightly increased (5-fold).

In the second set of mutations, the three histidines, -271, -332, and -334, were substituted for alanine in order to leave the possibility for hydrophobic interactions involving the methyl group while suppressing the polar ones due to the imidazole ring. As shown in Table II, substituting His-271 for Ala only yielded a 25% decrease in the rate of tRNA charging whereas there was no detectable effect on the kinetic parameters when this change was made at position 332. On the contrary, the replacement of His-334 by Ala had the same effects as its substitution for Gly.

The third series of mutagenesis experiments consisted of the replacement of each of these three histidines by glutamine whose side chain has hydrogen-bonding potentials similar to that of the imidazole ring. The mutation His-271 → Gln had virtually the same consequences as the previous replacement (His-271 → Ala) whereas substituting His-332 for Gln was totally harmless. As was the case in the second series, the mutation His-334 → Gln had quite different consequences: the overall rate of tRNA aspartylation was not changed as compared to the native enzyme, but there was a 10-fold decrease in the rate of the ATP–PP_i exchange reaction together with a slight but significant increase in the K_m values for the two small substrates (6-fold for L-Asp and 10-fold for ATP). As to the last change (His-334 → Arg), it was introduced in view of the recent discovery of a second class of aminoacyl-tRNA synthetases sharing three structural motifs (Eriani et al., 1990). Its effects on enzyme activity will be discussed in the next section.

Taken together, all these results show that His-271 and His-332 must play a structural rather than catalytic role since their replacement by side chains, offering some possibilities for hydrophobic or polar interactions, does not cause any drastic changes in the enzyme binding capacities and catalytic activities. On the other hand, His-334 seems to be part of the active site since any of the three mutations introduced at this position yields a mutated enzyme with altered binding and/or catalytic properties.

Finally, tyrosine-195 and three of the four lysines, -142, -151, and -382, labeled by DEPC were substituted for glycine. Lys-77 was not touched since the first 90 residues of aspartyl-tRNA synthetase are not essential for its activity (J. Gangloff, personal communication). The mutation Tyr-195 → Gly had no consequence at all whereas the replacement of each of these three lysines only caused a small decrease in the rate of tRNA aspartylation (data not shown). This favors a structural role for these three lysines but not their involvement in the active site. This last result is in agreement with those of DEPC labeling: indeed, the aminoacylation activity was

restored after hydroxylamine hydrolysis, which regenerates free imidazole and phenol groups while leaving a stable N^{ϵ}-carbethoxylysine side chain.

Discussion

The chemical modification used in this work has at least two well-known major drawbacks: the lack of selectivity of the reagent and the relative unstability of the *N*-carbethoxyimidazole arising from the reaction with histidine (and the same is true for tyrosine as well). Indeed, a priori, three different types of amino acids can react with DEPC: histidine, tyrosine, and lysine (only the latter giving rise to a stable product). The conditions used in this study and particularly the large excess of reagent with regard to histidine residues undoubtedly increase the risks of nonspecific side reactions, but this was a deliberate choice in order to achieve a maximal yield of labeling for these residues. It must be emphasized that under these conditions the three expected types of modified amino acids, histidine, tyrosine, and lysine, were actually found in the major radioactive peptides isolated from the chymotryptic digest of aspartyl-tRNA synthetase after its inactivation by radioactive DEPC. Moreover, their total number (8) is close to that of DEPC molecules incorporated into each subunit when virtually complete inactivation was reached (6–7 mol of DEPC per monomer at pH 6). This relatively high number strongly suggests that the chemical reagent reacted not only with residues contributing to the active site or to the maintaining of an active enzyme conformation but also with outsiders as well. In other words, there was no such competitive or preferential labeling of active-site residues due to an enhanced reactivity toward the chemical probe. Yet the recovery of activity following hydroxylamine hydrolysis and subsequent regeneration of free histidine and tyrosine from the corresponding labeled residues indicated that those lysines irreversibly modified by DEPC were not involved in either substrate binding or catalysis and this was confirmed by the results of site-directed mutagenesis: substituting each of the modified lysines (142, 151, and 382) for glycine had little effect on the enzyme kinetic parameters.

The unstability of the *N*-carbethoxyimidazole compound also poses a serious problem in that some of the labeled histidines may well have escaped detection despite the short chymotryptic digestion and the fast HPLC isolation procedure. Indeed, when a major radioactive peak contained a peptide mixture including one with a modified histidine, it was often noticed that a second purification step on the same reverse-phase column partly yielded the form with free histidine from the modified species (the labeled peptide and the intact one usually had different retention times). To overcome this difficulty, it seemed essential not to limit the site-directed mutagenesis experiments to those three histidines that were actually found to be labeled by DEPC (residues 190, 334, and 375) but to extend this study to all of them. This did not look too difficult a task since aspartyl-tRNA synthetase contains 12 histidines per subunit.

In order to minimize the number of experiments, it was first decided to replace each of these 12 histidines by glycine. It was assumed that if an imidazole group is directly involved in either substrate binding or catalysis or in maintaining an active three-dimensional structure via ionic interactions or hydrogen bonding with other partners, its replacement by a residue devoid of any side chain should have a strong destabilizing effect and should result in significant alterations of the kinetic parameters. The results of this first set of mutations clearly showed that only those three affecting His-271, His-332, and His-334 yielded such alterations. For the other nine histidines, the mutation His → Gly had little or no consequences, indicating that they are not part of the substrate binding or catalytic residues. Nor do they play a crucial role in maintaining an active enzyme conformation.

In the next series of mutagenesis experiments, the attention was focused on those three histidines, 271, 332, and 334, whose replacement by glycine significantly changed the enzyme properties. The two substitutions introduced at each position were selected, one (alanine) because it leaves a smaller side chain still able to make some hydrophobic contacts with neighboring residues and the other one (glutamine) because the γ-amide group can act as a donor or acceptor in hydrogen bonding as does the imidazole ring. The results gathered in Table II clearly show that neither His-271 nor His-332 can be directly involved in the binding of the small substrates (L-Asp and ATP) or catalysis since their replacement by either Ala or Gln does not inactivate the enzyme and does not change the K_m values to an appreciable extent. Rather, it looks as though these two residues play some important structural role by making hydrophobic contacts and/or hydrogen bonds with neighboring partners, thus actively participating in the building up of the enzyme three-dimensional structure. This idea is supported by comparison with other known aspartyl-tRNA synthetases (Mirande & Waller, 1989; Jacobo-Molina et al., 1989; Gampel & Tzagoloff, 1989; Eriani et al., 1990): indeed, His-271 is replaced by Gln in the rat liver and human enzymes and by Glu in their *E. coli* and yeast mitochondrial counterparts. As to His-332, it is conserved in the eukaryotic enzymes but replaced by Ala in the bacterial and yeast mitochondrial species. Furthermore, it has been recently proposed that aspartyl-tRNA synthetase belongs to a second class of enzymes that do not contain either of the two conserved HIGH or KMSKS sequences (Eriani et al., 1990): this class II gathers aspartyl-, asparaginyl-, threonyl-, seryl-, prolyl-, histidyl-, lysyl-, and the small subunit of phenylalanyl-tRNA synthetases (and perhaps glycyl- and alanyl-tRNA synthetases as well?). Very recently, the three-dimensional structure of seryl-tRNA synthetase has been published (Cusack et al., 1990): it does not contain the typical Rossmann's fold identified in the three already known three-dimensional structures belonging to class I (Zelwer et al., 1982; Brick et al., 1988; Rould et al., 1989) but a central β sheet made of eight antiparallel strands (and two additional α helices).

A comparison including 19 sequences from various sources indicates that enzymes from class II share 3 common motifs, and it is interesting to note that His-271 belongs to the first one while His-332 and -334 are part of the second one (Eriani et al., 1990). His-271 is not at all conserved and can be replaced by either Gln or Glu as mentioned above but also by various residues, Asp, Asn, Ser, Gly, Ile, Tyr, Lys, or Arg, and similarly various substitutions occur at position 332 (second motif): Ser, Gln, Ala, Val, Met, Leu, Lys, and Arg. The situation is strikingly different for position 334 at which only *His* (9 times) or *Arg* (10 times) were found. Therefore, the presence of a basic side chain seems to be essential at this position, and this immediately suggests a polar interaction between this positively charged residue and the pyrophosphate moiety of ATP as already shown for His-45 of tyrosyl-tRNA synthetase (Jones et al., 1986). The results given in Table II support such a hypothesis: indeed, substituting His-334 for either Gly or Ala yields a mutated enzyme with an increased K_m value for ATP (6–7-fold) and decreased velocities for both ATP–PP_i exchange and tRNA aminoacylation reactions. Surprisingly, the latter reaction is not affected by the mutation His-334 → Gln that gives rise to an enzyme with increased

K_m values for the small substrates (10-fold for ATP and 6-fold for L-Asp) and a reduced ATP–PP_i exchange reaction rate. When the naturally occurring change (His-334 → Arg) was introduced into the yeast enzyme, the ATP–PP_i exchange activity and the K_m value for ATP were those of the wild type, but there was a 10-fold decrease in the K_m value for aspartic acid and the aminoacylation activity was still reduced (25% as compared to the wild type). At this stage, it is difficult to interpret these puzzling findings, but clearly, His-334 must occupy a critical position in the three-dimensional structure of the yeast enzyme, thus allowing the polar imidazole ring to interact most probably with the pyrophosphate moiety of ATP and perhaps with the 3′ end of $tRNA^{Asp}$ as well. In the recently published three-dimensional structure of seryl-tRNA synthetase from *E. coli*, the amino acid corresponding to His-271 is Tyr-194, the last residue of the last β-strand A8 (Cusack et al., 1990). It is in fact located at the very end of the central β sheet believed to harbor the active site. On the other hand, the two residues equivalent to His-332 and -334, Leu-281 and Arg-283, respectively, are part of a poorly resolved loop in the middle of this β sheet between strands A7 and A6 (Cusack et al., 1990).

All these data bring some credibility to the idea that His-334 is part of the active site of aspartyl-tRNA synthetase from *Saccharomyces cerevisiae*. The recent progress in the crystallographic work done in this institute should soon bring a detailed map of the enzyme–tRNA complex. Together with this structural approach, an extensive kinetic study of the wild-type and mutated enzymes must be undertaken to answer the questions raised by the results reported here.

ACKNOWLEDGMENTS

We thank Professor J. P. Ebel for his constant help and interest and the Société Transgène (Strasbourg, France) for giving both the *E. coli* TGE strain and the expression vector pTG 900. The skillful technical assistance of Ms. Monique Le Ret, Annie Hoeft, and Marie-Louise Gangloff is gratefully acknowledged.

Registry No. L-His, 71-00-1; L-Lys, 56-87-1; aspartyl-tRNA synthetase, 9027-32-1.

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