Photoaffinity Labeling with the Activator IMP and Site-Directed Mutagenesis of Histidine 995 of Carbamoyl Phosphate Synthetase from *Escherichia coli* Demonstrate That the Binding Site for IMP Overlaps with That for the Inhibitor UMP†

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ABSTRACT: Photoaffinity labeling with IMP was used to attach covalently this activator to its binding site of *Escherichia coli* carbamoyl phosphate synthetase. We now identify histidine 995 of the large enzyme subunit as the amino acid that is cross-linked with IMP. The identification was carried out by comparative peptide mapping in two chromatographic systems of peptides differentially labeled with [^{3}H]IMP and with the labeled inhibitor [^{14}C]UMP, followed by automated Edman degradation and radiosequence analysis. Site-directed substitution of His995 by alanine confirmed His995 to be the only amino acid in the protein forming a covalent adduct with IMP. The His995Ala mutant protein was soluble and active and exhibited normal kinetics for the activator ornithine and for the substrates in the presence of ornithine. However, the mutation selectively induced changes in the activation by IMP and the inhibition by UMP, and it abolished the photolabeling of the enzyme by IMP without affecting the photolabeling by the inhibitor UMP. Since UMP is cross-linked to Lys993 [Cervera, J., et al. (1996) *Biochemistry 35*, 7247−7255] only two residues upstream of the site of IMP labeling, the results provide structural evidence for earlier proposals which suggested that UMP and IMP bind in a single or overlapping site. The two residues are within the region previously proposed as the binding fold for the nucleotide effectors. In the crystal structure of the enzyme, Lys993 and His995 are exposed and line a crevice where a P_i molecule was found [Thoden, J. B., et al. (1997) *Biochemistry 36*, 6305−6316]. UMP and IMP appear to bind in this crevice, possibly toward the C-side of the β-sheet in a Rossman fold. Their binding in this site is consistent with the selectivity of adduct formation of UMP with Lys993 and of IMP with His995. It is also consistent with the nonessentiality of His995 for the binding, since the interactions with other residues that line the crevice must contribute a large part of the binding energy. The lack of an effect of the mutation on the activation by ornithine is consistent with the binding of this activator in a separate site in the protein.

Carbamoyl phosphate synthetase catalyzes the formation of an essential precursor (carbamoyl phosphate) of arginine and pyrimidine biosynthesis. In *Escherichia coli* and most bacteria, a single carbamoyl phosphate synthetase provides carbamoyl phosphate for both pathways. The enzyme is highly feedback inhibited by the pyrimidine UMP and is activated by the purine IMP (*1*) and by the precursor of arginine, ornithine (*2*). *E. coli* carbamoyl phosphate synthetase is a large protein with a complex multidomain structure. The enzyme is a heterodimer of 40 and 120 kDa subunits (reviewed in ref *3*). The smaller subunit cleaves glutamine, generating the ammonia used by the larger subunit in the synthesis of carbamoyl phosphate according to the reaction

$$NH_3 + 2ATP + HCO_3^- \rightarrow 2ADP + P_i + \text{carbamoyl phosphate}$$

The larger subunit also binds the effectors. This subunit consists of two homologous halves (*4*) associated as a complementary isologous pseudohomodimer (*5*, *6*). Each homologous half is composed of a proximal 40 kDa region that resembles structurally the biotin carboxylase component of biotin enzymes and that is involved in binding an ATP molecule and in catalysis, and a distal 20 kDa domain (*5*, *6*). The latter domain in the C-terminal half of the subunit binds the inhibitor UMP (*5*) and the activator IMP (*7*), which influence the activity of the enzyme at both ATP sites. This domain is also involved in allosteric control in the carbamoyl phosphate synthetases from other organisms and is generally considered to be the regulatory domain (*8*, *9*). In *E. coli*

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carbamoyl phosphate synthetase, there is evidence from structure−reactivity (*10*, *11*) and kinetic (*12*) studies which suggests that the inhibitor UMP and the activator IMP bind to the same region of the protein at overlapping sites. Such binding presumably leads to two different types of conformational change in the enzyme, resulting in inhibition or activation. However, the nature of the conformational changes and the structural characterization of the sites for these effectors have not yet been established. Photoaffinity labeling studies with [^{14}C]UMP and comparative sequence analysis localized the site of UMP binding when the sequence of a peptide containing the UMP label was determined and showed that the uridine nucleotide was covalently linked with Lys993[1] (*13*). Site-directed mutagenesis (*14*) and modeling (*6*) studies suggest that Thr977 is an essential residue involved in UMP regulation of the enzyme. The results of these investigations provide evidence for the location of a nucleotide binding fold in the region surrounding Lys993 in the C-terminal domain of the enzyme large subunit (*13*). In the studies described here, the regulatory nucleotide binding site was further characterized, and we provide structural evidence of an overlapping site for UMP and IMP with the demonstration that the photoaffinity labeling with IMP results in the cross-linking of this activator to a single residue, His995, located in the C-terminal regulatory domain. Site-directed replacement of His995 with alanine selectively affected modulation of the enzyme by IMP and UMP, and the ability of the mutant protein to be photoaffinity labeled with IMP was lost.

EXPERIMENTAL PROCEDURES

Chemicals and Enzymes. $Na_2{}^{14}CO_3$ (56 mCi/mmol), [α-^{35}S]-dATPαS (1000 Ci/mmol), and [2-^{3}H]AMP (21 Ci/mmol) were from Amersham. [2-^{3}H]IMP (21 Ci/mmol) was prepared from [2-^{3}H]AMP with 5′-adenylic acid deaminase and was used as described for photoaffinity labeling of the IMP site of carbamoyl phosphate synthetase (*7*). [2-^{14}C]UMP (46.1 mCi/mmol; from Sigma España) was used for labeling the UMP site (*5*). Other reagents and enzymes, including carbamoyl phosphate synthetase from *E. coli*, were obtained or prepared as previously described (*13*). The labeled 25.8 kDa C-terminal fragment of the large subunit was generated with V8 staphylococcal protease and was isolated by HPLC[2] as reported previously (*5*).

Cyanogen Bromide Cleavage. CNBr cleavage of the [^{14}C]-UMP-labeled 25.8 kDa C-terminal fragment of the carbamoyl phosphate synthetase large subunit, and analysis of the fragments by SDS−urea−PAGE and fluorography, was carried out exactly as described previously (*13*). The [^{3}H]-IMP-labeled carbamoyl phosphate synthetase (80 000 cpm/mg of protein) was subjected to CNBr cleavage in the same way, except for the omission of the HPLC step used to isolate the 25.8 kDa [^{3}H]IMP-labeled C-terminal fragment. In this case, after termination (by boiling) of the step of limited proteolysis with V8 staphylococcal protease that releases the [^{3}H]IMP-labeled 25.8 kDa fragment, the protein was precipitated with cold acetone (*15*) and was then subjected to CNBr digestion.

Peptide Mapping with Different Proteases. The material to be digested, consisting of approximately equiradioactive (1000−25000 cpm) mixtures of the [^{3}H]IMP- and [^{14}C]UMP-labeled 25.8 kDa C-terminal fragment or of peptides generated by digestion of this fragment with specific proteases (see the Results), was lyophilized in a sterile tube and was dissolved in the sterilized (by either filtration or boiling) digestion buffer. The digestion was started by the addition of the protease and was continued for 22 h at 25 °C (except where indicated), with another addition of the protease after 11 h. Specific digestion conditions are given in the legend of Figure 2. Digestions were terminated by injection of the mixture into an HPLC reversed phase Vydac 5 mm C-18 column (type 218TP54, 0.46 cm × 25 cm), followed by elution with a 60 min linear gradient (9.5 to 38%) of acetonitrile, in the presence of 0.1% trifluoroacetic acid, at a flow rate of 1 mL/min. Fractions were collected at 1 min intervals, and the amount of ^{3}H and ^{14}C radioactivity was determined in the fractions by dual-isotope liquid scintillation counting. The radioactive material remained unchanged (shown by HPLC) in control incubations without protease. Mixtures of the [^{3}H]IMP- and [^{14}C]UMP-labeled material from the fractions, and of GnRH, RNAase A, and BSA, were subjected to gel filtration through Sephacryl S-200 in the presence of SDS as previously described (*13*), to separate the products of peptide mapping on the basis of their masses.

Automated Solid Phase Edman Degradation of the Products of Peptide Mapping. The separate [^{3}H]- and [^{14}C]peptide fractions eluted from the reversed phase column were mixed to give approximately equal amounts of ^{3}H and ^{14}C in the sample to be analyzed. The labeled peptides (1000−20000 total cpm) were lyophilized; the residue was dissolved in 35−50 μL of 70% formic acid, and the sample was applied to a Sequelon AA membrane (from Millipore). Treatment of the membrane with carbodiimide and automated sequential Edman degradation were carried out as recommended by the manufacturer for N-terminal sequencing with the solid phase Pro-sequencer apparatus (Milligen, Millipore), the only modification being that the effluent from each degradation cycle was collected to determine the amount of ^{3}H and ^{14}C radioactivity by dual-isotope liquid scintillation counting.

Preparation of the Mutant His995Ala. Standard protocols were used for transformation of *E. coli* and for isolation, digestion, and ligation of DNA (*16*). Plasmid DNA from pLLK12 (*17*) (which contains the *car*AB gene inserted into the *Bam*HI site of pUC19) was digested with *Sma*I, and the DNA fragment (897 bp) encoding the C-terminal region of *car*B was isolated electrophoretically and was ligated with the *Sma*I site of bacteriophage M13mp19. Site-directed mutagenesis was carried out according to ref *18*, using uracil-containing single-stranded DNA generated in *E. coli* strain CJ236 (*18*) and the antisense oligonucleotide 5′-GACGGC-CTTCAGCCACCTTGTTTAC-3′, in which the underlined triplet (complementary to the GCT codon for alanine) replaces the original triplet ATG (complementary to the CAT of codon 995 of *car*B, encoding histidine). The mutation was corroborated by manual sequencing (*19*) of single-stranded

[1] In a previous publication (*13*), Lys993 was incorrectly identified as Lys992. This has been corrected in this publication, and Lys993 now corresponds to codon 993 in the nucleotide sequence of the *E. coli car*B gene.

[2] Abbreviations: BSA, bovine serum albumin; CNBr, cyanogen bromide; HPLC, high-performance liquid chromatography; PAGE, polyacrylamide gel electrophoresis; PRPP, phosphoribosyl pyrophosphate; SDS, sodium dodecyl sulphate; Tris, tris(hydroxymethyl)-aminomethane.

DNA isolated from individual colonies of DH5α cells (from Clontech) transformed with the M13 vector containing the mutated insert.

The mutant *car*AB gene was constructed by replacement of the wild type *Sma*I fragment of *car*B with the corresponding mutant fragment carrying the CAT → GCT mutation. The resultant plasmid pLLK12H995A was used to transform *E. coli* strain L814 (*car*AB$^-$) (*17*), and the presence of the mutation in the final construct was again confirmed by sequence analysis of the mutant plasmid isolated from single colonies of L814 transformants. The mutant carbamoyl phosphate synthetase encoded by pLLK12H995A was prepared from the overproducing L814 strain and purified (95% homogeneity, as judged by SDS−PAGE) as the wild type enzyme.

Enzyme Activity Assays. Carbamoyl phosphate synthetase activity was measured by the extent of incorporation of $H^{14}CO_3^-$ into [^{14}C]carbamoyl phosphate, which was determined at the end of the assay as the amount of [^{14}C]urea (*20*). Assays were carried out at 37 °C in closed Eppendorf 1.5 mL tubes containing 50 μL of a solution of 0.1 M Tris-HCl (pH 8), 0.1 M KCl, 10 mM glutamine, 1 mM ATP, 1 mM $MgCl_2$, 10 mM $NaH^{14}CO_3$ ($1-3 \times 10^3$ cpm/nmol), and the indicated concentration of either ornithine, UMP, or IMP. Alternatively, 10 mM ornithine was included in the assays, and the concentrations of individual substrates were varied. When the ATP concentration was varied, the $MgCl_2$ concentration was kept equimolar with that of the nucleotide. The effects of variable concentrations of ammonium ion (added as NH_4Cl) were assayed in the absence of glutamine. Incubations were terminated after 10 min by transfer to ice, followed by the addition of 0.05 mL of 3 M KOH and 0.05 mL of 1 mM carbamoyl phosphate. Conversion of carbamoyl phosphate to urea and determination of the amount of [^{14}C]urea, free from $H^{14}CO_3^-$, were carried out as previously described (*20*).

RESULTS

Mapping of the IMP Site by Cleavage with CNBr. We previously reported (*7*) the specific photoincorporation of IMP in the large subunit of *E. coli* carbamoyl phosphate synthetase within the 25.8 kDa C-terminal fragment (residues Val837−Lys1073) that is generated by limited proteolysis with V8 staphylococcal protease. Further mapping studies revealed that the site of labeling was located downstream of the specific tryptic cleavage site at Arg912 (*7*). According to the known sequence of carbamoyl phosphate synthetase, the 25.8 kDa fragment contains six methionines located at positions 869, 911, 918, 939, 1052, and 1068 (*4*), and consequently, cleavage of the 25.8 kDa fragment with CNBr should generate a 12.6 kDa fragment (residues 940−1052) and six <5 kDa fragments. The electrophoretic migration on SDS−urea−PAGE of the radioactivity after CNBr cleavage of the [^{14}C]UMP- or [^{3}H]IMP-labeled 25.8 kDa C-terminal fragment (Figure 1) confirms (*13*) that UMP is cross-linked to the 12.6 kDa fragment and shows that IMP is cross-linked to the same fragment (Figure 1), which was previously characterized by N-terminal sequencing (*13*) as the fragment beginning with Lys940.

Comparative Peptide Mapping and Radiosequencing of the IMP Site. When a mixture of the [^{14}C]UMP- and [^{3}H]-IMP-labeled C-terminal 25.8 kDa fragments was digested exhaustively with V8 protease and the mixture was analyzed by reversed phase HPLC, the resulting ^{3}H- and ^{14}C-labeled peptides were eluted as single narrow overlapping peaks differing in their maxima by only 1 min (Figure 2, HPLC). The near coincidence of the two isotopes would be expected if the chromatographic behavior of the same minimal V8 peptide were modified slightly differently when it is cross-linked to UMP or to IMP. The [^{3}H]IMP- and [^{14}C]UMP-labeled peptides have essentially the same mass, as judged from their identical behavior on a second chromatographic system consisting of gel filtration through Sephacryl S-200 in the presence of SDS (Figure 2, Sephacryl), a system that separates peptides according to their size (*13*, *21*). This finding also supports the cross-linking of UMP and IMP to the same minimal V8 peptide. The sequence of the V8 minimal peptide carrying the UMP label is known from previous work (*13*) to be ^{984}AGINPRLVNKVHE. UMP is cross-linked to Lys993, located at position 10. In the studies presented here, the site of UMP labeling was verified by radiosequencing analysis. When a mixture of the [^{14}C]UMP- and [^{3}H]IMP-labeled V8 peptides isolated by HPLC (fractions 26 and 27) was subjected to radiosequencing in an automated solid phase N-terminal sequenator,[3] the ^{14}C radioactivity was eluted during cycle 10 of the degradation (Figure 3), confirming the identity of the V8 peptide and Lys993 as the site of UMP labeling. Tritium label was eluted during cycle 12 of degradation (Figure 3). If, as it appears, IMP is cross-linked with an identical V8 peptide, then His995 must be the site of IMP labeling.

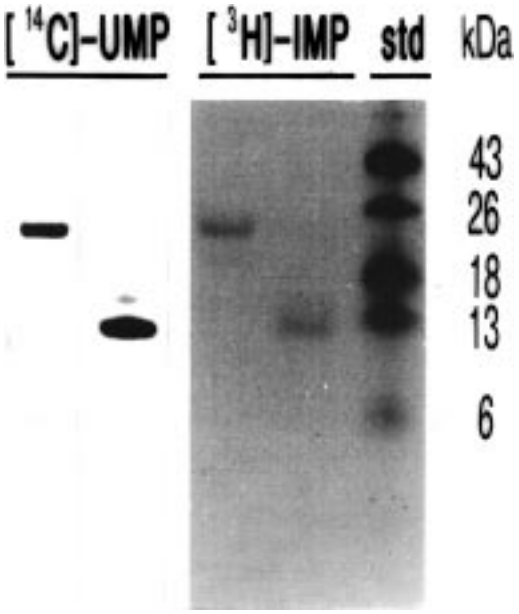

FIGURE 1: CNBr cleavage of the [^{14}C]UMP- or [^{3}H]IMP-labeled 25.8 kDa fragment, analyzed by SDS−urea−PAGE and fluorography. For both [^{14}C]UMP and [^{3}H]IMP, the left track illustrates the undigested control and the right track the products of digestion. The masses of standard proteins are shown to the right. For further details, see Experimental Procedures.

[3] The use of a solid phase sequenator was essential for radiosequence analysis. The polarity of the solvents is higher than with the gas phase sequenator, resulting in the elution of the radioactive nucleotide−amino acid photoadducts. These were retained in the membrane in gas phase sequenators.

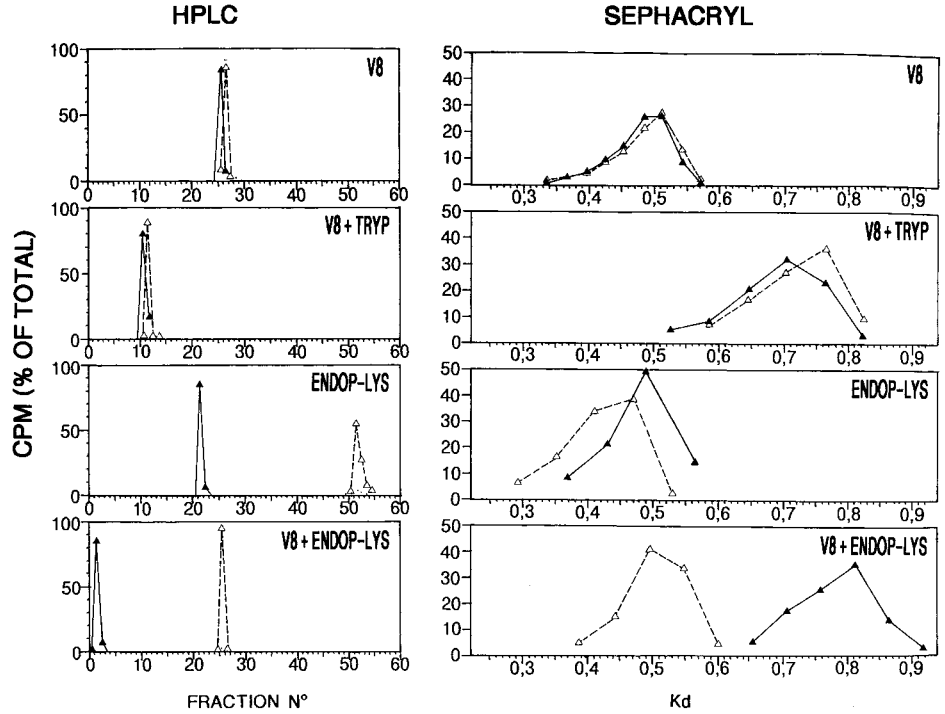

FIGURE 2: HPLC and gel filtration analysis of the proteolytic digestion products of the [^{3}H]IMP- and [^{14}C]UMP-labeled 25.8 kDa fragment and of fragments thereof. White and black symbols denote radioactivity due to ^{14}C and ^{3}H, respectively. V8, digestion of the 25.8 kDa fragment with 50 μg/mL V8 staphylococcal protease in 50 mM ammonium bicarbonate (pH 7.8) and 2 M urea; V8+TRYP, digestion of the radioactive products (fractions 26 and 27 of the HPLC system) of the prior V8 digestion with 20 μg/mL trypsin (TPCK-treated) in 0.1 M ammonium bicarbonate, 2 M urea, and 20 mM methylamine, at 35 °C; ENDOP-LYS, digestion of the 25.8 kDa fragment with 10 μg/mL endoproteinase Lys-C in 25 mM Tris-HCl (pH 8.5), 2 M urea, 20 mM methylamine, and 1 mM EDTA; and V8+ENDOP-LYS, digestion of the radioactive products (fractions 26 and 27 of the HPLC system) of the prior V8 digestion with 25 μg/mL endoproteinase Lys-C in 25 mM Tris-HCl (pH 8.5), 1 mM EDTA, 10% acetonitrile, and 0.1% trifluoroacetic acid. To facilitate comparison, the radioactivity in the fractions is expressed, for each isotope, as a percentage of the total amount of ^{3}H or ^{14}C radioactivity eluted from the column. K_d is the distribution coefficient, equaling $(V_e - V_o)/(V_i - V_o)$, taking V_o, V_i, and V_e to be the volumes of elution of BSA, mercaptoethanol, and the compound of interest, respectively. For further details, see Experimental Procedures.

The labeled V8 peptides (fractions 26 and 27 of HPLC) were further characterized by secondary cleavage with trypsin, as described in the legend of Figure 2. The tryptic treatment changed similarly the chromatographic behavior of the ^{3}H and ^{14}C radioactivity in the two separation systems used here. The two isotopes appeared in the HPLC system as neighboring peaks eluting much earlier than the parental V8 peptides, and coeluted much later than the parental peptides in the Sephacryl S-200 system (Figure 2, HPLC and Sephacryl). These results reflect a large decrease in the size of the labeled fragments, caused by the tryptic treatment, and are the results expected for the cross-linking of UMP and IMP with the same peptide. The UMP-labeled peptide generated after sequential exhaustive V8 and tryptic treatments is known from previous work (*13*) to be the peptide ^{990}LVNKVHE. Radiosequencing of the combined ^{3}H- and ^{14}C-labeled tryptic fragments (fractions 11 and 12 in the HPLC system) released (Figure 3) the ^{14}C during cycle 4, as expected for Lys993, and the ^{3}H during cycle 6, as expected if IMP were cross-linked to His995.

An independent confirmation that IMP is cross-linked to His995 was obtained using endoproteinase Lys-C. Upon treatment with this protease of a mixture of the [^{14}C]UMP- and [^{3}H]IMP-labeled C-terminal 25.8 kDa fragments, the ^{3}H was eluted much earlier in the HPLC system and considerably later in the gel filtration system than the ^{14}C (Figure 2). These results indicate that UMP and IMP are cross-linked to different endoproteinase Lys-C fragments, and that the IMP-labeled fragment is smaller than the UMP-labeled fragment. The results are those expected if IMP and UMP are cross-linked to His995 and Lys993. These residues would be the 2nd and 27th, respectively, of the 13-residue and 27-residue minimal endoproteinase Lys-C peptides ^{994}VHEGRPHIQDRIK and ^{967}QGFELDATHGTAIVLGEAGINPRLVNK, respectively. If these expectations were correct, radiosequencing of a mixture of the ^{3}H- and ^{14}C-labeled endoproteinase Lys-C peptides should release ^{3}H during the second cycle whereas ^{14}C should not be released during the initial 15 cycles of automated Edman degradation. The experimental results fully corroborate these expectations (Figure 3).

To confirm further the identification of His995 as the site of cross-linking of IMP, we subjected a mixture of the [^{3}H]-IMP- and [^{14}C]UMP-labeled V8 minimal fragments to exhaustive digestion with endoproteinase Lys-C. After such treatment, His995 and Lys993 should be the 2nd and 10th residues of two different peptides, ^{994}VHE and ^{985}AGINPRLVNK, respectively. In agreement with these predictions, the ^{3}H was eluted very early in the HPLC system and very late in the gel filtration system, whereas the ^{14}C was eluted in both systems at a position consistent with a larger peptide (Figure 2). Furthermore, cyclic Edman degradation released according to expectations the ^{3}H during cycle 2 and the ^{14}C during cycle 10 of the degradation cycle (Figure 3). In fact, since the automatic Edman degradations of the peptides generated with V8 protease, trypsin, and endoproteinase-LysC released the ^{3}H label during the 12th, 6th, and 2nd degradation cycles, respectively, IMP is cross-linked to a

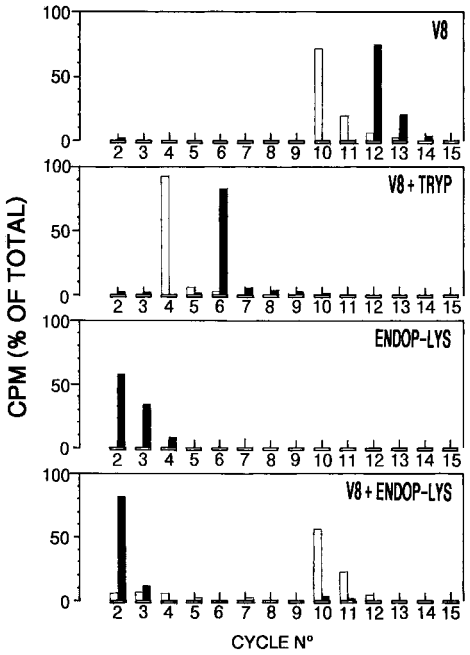

FIGURE 3: Yield of ^{3}H (filled bars) and ^{14}C radioactivity (unfilled bars) upon automatic Edman degradation of the products of peptide mapping. The following mixtures of ^{3}H- and ^{14}C-labeled peptides isolated by HPLC (Figure 2) were applied to the membrane: V8, fractions 26 and 27 of the HPLC system after digestion with V8 staphylococcal protease; V8+TRYP, fractions 11 and 12 after sequential digestion with V8 staphylococcal protease and trypsin; ENDOP-LYS, fractions 22 and 52 after digestion with endoproteinase Lys-C; and V8+ENDOP-LYS, fractions 2 and 26 after sequential digestion with V8 staphylococcal protease and endoproteinase Lys-C. To facilitate comparison, the amount of radioactivity in the fractions is expressed, for each isotope, as a percentage of the total ^{3}H or ^{14}C radioactivity eluted in all the cycles, except cycle 1, in which variable amounts of radioactivity appeared in all cases possibly due to elution of unfixed sample.

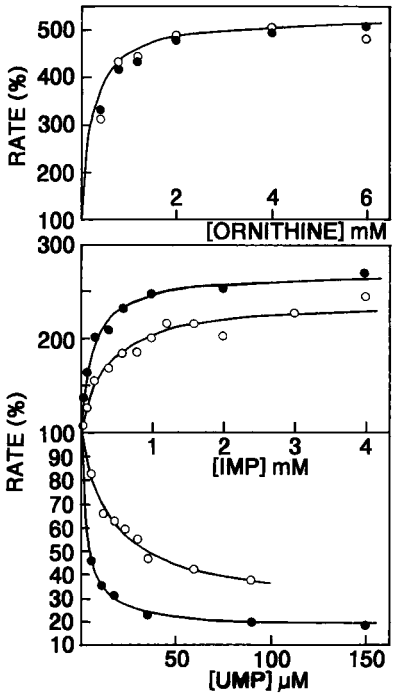

FIGURE 4: Effect of His995Ala substitution on the allosteric modulation of carbamoyl phosphate synthetase: (●) wild type enzyme and (○) His995Ala mutant enzyme. Velocities are expressed as a percentage of the velocity in the absence of effectors. Lines are hyperbolas defined by the following apparent parameters: origin, 100; ornithine, $V_{max} = 530$, $K_a = 0.23$ mM; IMP (wild type), $V_{max} = 270$, $K_a = 0.16$ mM; IMP (mutant), $V_{max} = 241$, $K_a = 0.37$ mM; UMP (wild type), $V^{[UMP]=\infty} = 17.4$, $K_i = 0.0032$ mM; UMP (mutant), $V^{[UMP]=\infty} = 25.7$, $K_i = 0.018$ mM.

residue that, given the specificities of these proteases, is 12, 6, and 2 residues downstream from glutamate, arginine/lysine, and lysine residues, respectively. In the CNBr fragment labeled with [^{3}H]IMP (residues 940−1052), His995 is the only residue fulfilling all three criteria (data not shown). Therefore, all the results shown thus far, taken jointly, conclusively show that IMP is photo-cross-linked to His995 of the large subunit.

Effect of the His995Ala Substitution on Allosteric Regulation and Photoaffinity Labeling. Since alanine is about 10-fold less efficient than histidine in forming photochemical adducts with nucleotide bases (*22*), we tested the effect of replacing His995 with alanine. The mutation had no substantial consequences on the expression, purification, or activity of the enzyme (determined in the standard assay), or on the substrate kinetics for ATP, bicarbonate, and either glutamine or ammonia, tested in the presence of 10 mM ornithine (data not shown). Similarly, the mutation did not change the concentration dependence of the activity for ornithine (Figure 4) or the specificity for the nucleotide effectors. As is the case with the wild type enzyme, with the mutant form UMP was an inhibitor and IMP an activator (Figure 4); 0.035 mM UTP, 0.1 mM PRPP, and 2 mM CMP did not influence the activity, and AMP was a less effective activator than IMP (data not shown). In contrast, the mutation selectively decreased 5.6- and 2.3-fold the apparent affinity for UMP and IMP, respectively, and by about 10% the maximal extent of inhibition and activation by these effectors (Figure 4). The selective effects of the mutation on the modulation by both nucleotide effectors and the lack of an effect on the modulation by ornithine are consistent with the binding of UMP and IMP in the same site and with the binding of ornithine in a physically independent site. The lack of substantial changes in the kinetic parameters for the substrates suggests that the mutation does not exert its effects by general changes in the conformation of the enzyme. Since UMP and IMP still have an effect on the mutant enzyme, histidine 995 is not an essential residue for binding of the nucleotide effectors.

Despite the nonessentiality of His995 for IMP binding, there was a complete loss of incorporation of IMP on photoaffinity labeling of the mutant enzyme with this nucleotide, whereas the labeling with UMP was little affected by the mutation. The fluorography results shown in Figure 5 illustrate the drastic decrease in the extent of [^{3}H]IMP labeling of the enzyme large subunit of the His995Ala mutant compared with that of the wild type enzyme. The faint radioactive band observed with the mutant enzyme and [^{3}H]-IMP is clearly due to nonspecific labeling, for the band is comparable to that observed with rat liver carbamoyl phosphate synthetase I (CPSI), an enzyme that is not activated by IMP, and is fainter than that observed with the wild type enzyme when [^{3}H]IMP is diluted with a 7.5-fold excess of unlabeled IMP. In contrast with the lack of labeling with IMP, the large subunit of the mutant enzyme was labeled with [^{14}C]UMP (Figure 5), whereas, again, no significant labeling was observed when an excess of unlabeled UMP was added or when carbamoyl phosphate

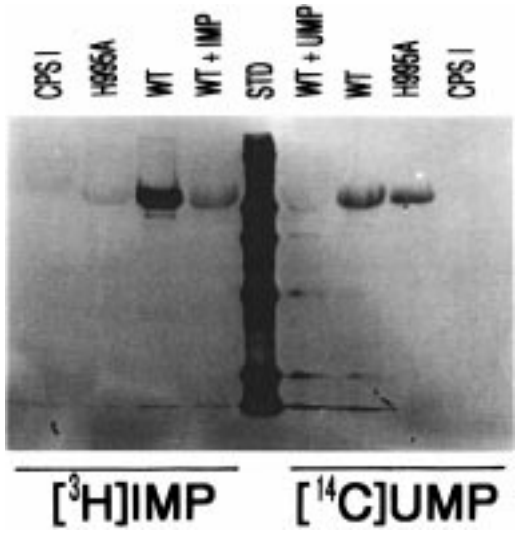

FIGURE 5: Effect of the His995Ala substitution on the photoaffinity labeling of carbamoyl phosphate synthetase by [^{3}H]IMP and [^{14}C]-UMP. The enzymes (2.5 mg/mL), wild type (WT) or mutant (H995A) *E. coli* carbamoyl phosphate synthetase, or rat liver mitochondrial carbamoyl phosphate synthetase (CPSI) was labeled with either 12 μM [^{3}H]IMP (*7*) or 9 μM [^{14}C]UMP (*5*). +IMP and +UMP denote the addition to the labeling mixture of 90 μM nonradioactive IMP or UMP before initiation of the UV irradiation. After irradiation, the mixtures were subjected to SDS−PAGE (10% polyacrylamide gel) and fluorography. Lane STD shows 200, 97.4, 69, 46, 30, and 14.3 kDa radioactive protein standards (from Amersham).

synthetase I was used. In summary, the results of these experiments confirm that His995 reacts specifically with IMP and exclude the involvement of this residue in the labeling with UMP.

DISCUSSION

The use of the inhibitor UMP and the activator IMP as specific allosteric site-directed photoaffinity labels has made it possible to identify specific residues (Lys993 and His995) in the binding sites for these effectors. Since under the conditions of labeling both UMP (*23*) and IMP[4] (*7*) are tightly bound with dissociation constants of <10 μM, the specificity of labeling is exceedingly high (*5*, *7*). Of the 1454 residues constituting the holoenzyme (see ref *3*), only a single amino acid, located in the C-terminal domain of the large subunit, was cross-linked with each of the two nucleotides. Like the normal noncovalent binding of these effectors, the formation of the covalent adducts with UMP or IMP protects the large subunit from cleavage by proteases (*5*, *7*), indicating that these effectors are cross-linked to their normal sites and cause the same conformational changes as the normally bound effectors.

Despite the high specificity, the usefulness of the labeling is limited by its efficiency. Although reasonable incorporation of UMP was achieved [approximately 5% (*13*)], only about 0.15% of the enzyme molecules incorporated IMP (*7*), because of to the lower intrinsic photoreactivity of the purine base (*22*). Thus, the IMP-labeled species was only present in trace amounts, which precluded the use of conventional amino acid sequence analysis, the most straightforward approach for identifying the site of modification. Nonetheless, the amount of specific radioactivity of IMP was sufficiently high to follow the label in peptide mapping and radiosequencing experiments. Knowledge of the amino acid sequence of the enzyme and the use of differentially UMP-labeled peptides as the internal standard for comparative peptide mapping and radiosequence analysis proved to be

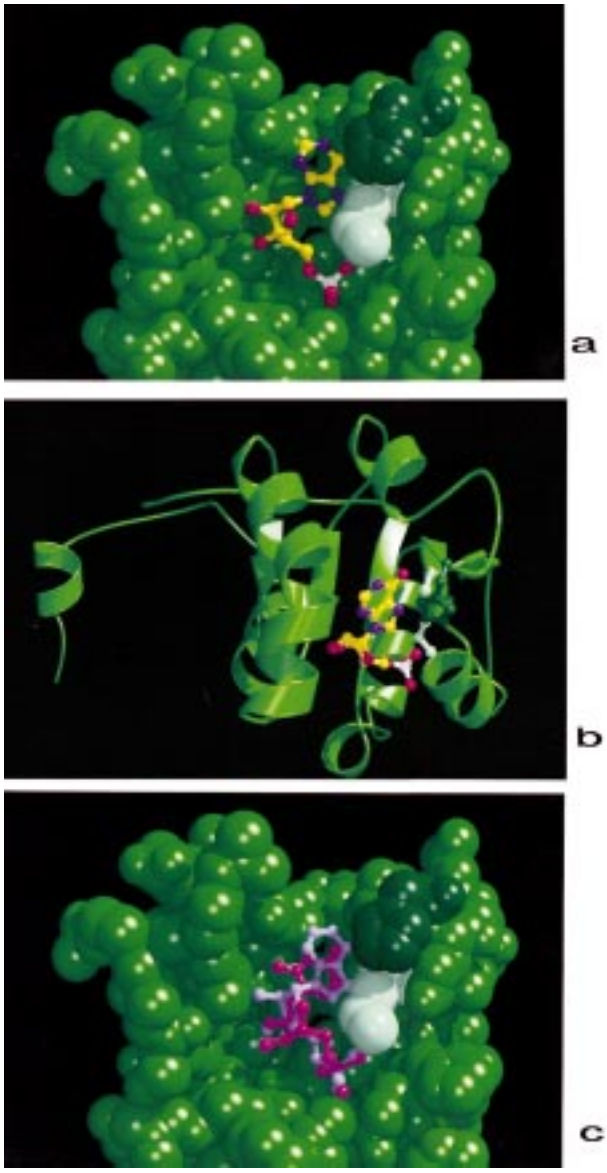

FIGURE 6: Putative nucleotide effector binding site. Panels a and c show space-filling representations of the site, with Lys993 colored white and His995 dark green. IMP and UMP are shown in ball-and-stick representations. In panel c, the two nucleotides are superimposed in the binding site. Panel b shows a ribbon representation of the putative binding site to illustrate the Rossman fold, with IMP shown in a ball-and-stick representation; the side chains of Lys993 and His995 are also shown explicitly in ball-and-stick representations. The model was built using program O and the coordinates deposited for carbamoyl phosphate synthetase (PDB file 1JDB) (*6*). IMP and UMP structures were taken from the library of heterocompounds of Uppsala University (http://alpha2.bmc.uu.se/hicup/). The nucleotides were positioned manually, fixing the phosphate at the position of the phosphate ion present in the reported enzyme structure (*6*), and keeping all atoms of the nucleotides a distance of at least 2 Å from any atom of the protein, to avoid potential steric problems.

[4] The conditions of the labeling assay were optimized previously (*7*) for high-affinity binding of IMP. The apparent affinity for IMP is lower under the conditions used in the activity assays.

```
Ec    947 LSVREGDKER VVDLAAKLLK QGFELDATHG TAIVLGEAGI-NPRLVNKVHE ----GRP----HIQDRIKNGEYTYIIN
St    947 LSVREGDKER VVDLAAKLLK QGFELDATHG TAIVLGEAGI-NPRLVNKVHE ----GRP----HIQDRIKNGEYTYIIN
Pa    946 ISVREDDKPF AAQVAGDLVA LGFEVVATAG TARVIEAAGL-PVRRVNKVTE ----GRP----HVVDMIKNDEVTLIIN
Mbo   976 VSVANRDKRS LVFPVKRLAD LGFRVLATEG TAEMLRRNGI-PCDDVRKHFE PAQPGRPT--MSAVDAIRAGEVNMVIN
Bs    940 LTVADKDKEE GLAIAKRFHA IGYNILATEG TAGYLKEASI-PAKVVGKIGQ ----DGP----NLLDVIRNGEAQFVIN
Bc    940 LTVADKDKEE AVELARRFAD IGYQLLATNG TAETLKAAGI-PVTVVNKIHS ----ASP----NILDVIRQGKAQVVIN
Lp    940 LTVRDEDKPE TVALAKRFHA LGYQLLATRG TATALTTHGL-PVTTVDKIDS ----GEH----DLLHRMEAGEVQVVIN
Ll    940 FTVADEDKEE TLALAKDFAE IGYSLVATAG TAAFLKENGL-YVREVEKLAG ----GEDEEG-TLVEDIRQQRVQAVVN
Mb        LSIRNADKTE LVDVAKKLQA AGLELMGTEG TVNYLAQHGV-FMDVVKKVHD ----GSP----NVIDMMRRDEVNLIIN
Ss    940 LVYGNKNLDY LKDTADNLTR FGLTVYSISE LPLQDIETID---------- ----------KMKAEELVRAKKVEIIV
Bs    933 VQNVPEDVKE LAENA-------GFTI--HEG T-------------------- ------------FASWMEQEGNSLHIN

Ssp   979 VSMSDRTKEA AVPVVRELID LGFKVVATSG TQKVLREHGIEGVEVVLKLHE ----GRP----HVIDWIKNGQIQFIIN
At    937 VFLNDMTKPH LEKIAVSFLE LGFKIVATSG TAHFLELKGI-PVERVLKLHE ----GRP----HAADMVANGQIHLMLI
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FIGURE 7: Alignment of plant and bacterial carbamoyl phosphate synthetase sequences in the region of the nucleotide effector binding fold. Lys993 and His995 along with other amino acids (K954, T974, and T977) that have been implicated in binding of the nucleotide effectors in the *E. coli* enzyme (*6*, *14*) are denoted in bold type. All sequences were obtained from GenBank. The reported sequence of the enzyme from *Methanosarcina barkeri* is a partial sequence of the C-half of the protein; therefore, the sequence is not numbered. The following abbreviations are used here: *Ec*, *E. coli*; St, *Salmonella typhimurium*; Pa, *Pseudomonas aeruginosa*; Mbo, *Mycobacterium bovis*; *Bs* and *Bc*, pyrimidine-specific enzymes from *Bacillus subtilis* and *Bacillus caldolyticus*, respectively; *Lp*, *Lactobacillus plantarum*; *Ll*, *Lactococcus lactis*; Mb, *M. barkeri*; Ss, *Sulfolobus solphataricum*; Ssp, *Synechocystis* sp.; and *At*, *Arabidopsis thaliana*.

critical for unambiguous identification of the site of IMP incorporation.

The results of radiosequence analysis have confirmed our previous conclusion (*13*) that UMP is cross-linked to Lys993. The analysis also revealed that IMP is cross-linked to His995, only two residues downstream of the site of labeling with UMP. The conclusion that His995 is the only amino acid in the protein that is responsible for incorporation of IMP was verified by site-directed substitution of the reactive histidine with alanine. The substitution had only a modest effect on the activation of the enzyme by IMP, but it completely abolished the ability of the protein to incorporate IMP, as anticipated, given the much lower susceptibility of alanine than of histidine to formation of photoadducts with nucleotide bases (*22*).

The fact that the reactive amino acids Lys993 and His995 are near neighbors in the amino acid chain provides direct structural evidence for the earlier proposal of Boettcher and Meister (*10*, *11*) who suggested that UMP and IMP bind at distinct amino acid residues located within a single or overlapping nucleotide binding site. This proposal was based on kinetic studies showing that UMP and IMP were mutually competitive with each other, and that both nucleotides were competitive with dUMP, which is neither an activator nor an inhibitor (*11*). These results, together with the observation that a dialdehyde derivative of UMP (*10*) or its arabinose analogue (*11*) is an activator rather than an inhibitor, led the authors to suggest that the pyrimidine and purine rings could potentially interact with different subsites located within a common nucleotide binding site (*11*), a prediction that agrees well with our findings presented here.

The uracil and hypoxanthine rings are the elements in UMP and IMP that absorb UV light of the wavelengths used here. Therefore, these rings are expected to be directly involved in the photochemical addition to the enzyme, and thus to be very close to the side chains of labeled amino acids Lys993 and His995. These results are consistent with the modeling of these nucleotides in the carbamoyl phosphate synthetase structure. IMP (Figure 6a) has appropriate dimensions for binding in a crevice found in the C-terminal domain that is lined by Lys993 (white) and His995 (dark green). The orientation of the IMP is such that the phosphate group occupies the position of the bound phosphate found in the enzyme crystal (*6*). As expected for the binding of a nucleotide, a Rossman fold exists at this site and IMP binds toward the C-side of the β-sheet (Figure 6b). The proximity of the hypoxanthine ring to the imidazole ring of His995 is obvious in panel a of Figure 6. In Figure 6c, IMP and UMP are superimposed in the same binding site to illustrate that the site can accommodate one nucleotide or the other and that the uracil ring would be much closer to Lys993 than to His995, consistent with the specificity of the photoaffinity labeling with each nucleotide. The existence of many interactions with the nucleotides, other than those due to His995, accounts for the relatively small effect of the replacement of His995 with alanine on the binding of the nucleotide effectors.

In any case, the finding that UMP and IMP occupy the same binding site lends further support to our previous proposal that the binding site for UMP is entirely located within the C-terminal domain in the sequences surrounding Lys993 (*13*). This lysine is generally conserved in the sequences of bacterial carbamoyl phosphate synthetases (Figure 7), consistent with a widespread occurrence of feedback inhibition by UMP in bacteria. In contrast, the His is conserved in the sequences of only about half of the same carbamoyl phosphate synthetases. Of these, only carbamoyl phosphate synthetase from *E. coli* and *Salmonella typhimurium* have been reported to be activated by IMP (*3*, *24*). However, in many bacteria the regulatory properties of carbamoyl phosphate synthetase are not known, particularly with respect to IMP activation. It is not possible at present, therefore, to correlate the presence of histidine with IMP activation. Nevertheless, it appears to be significant that the amino acids corresponding to Lys993, His995, Thr974, Thr977, and Lys954 are also found in the sequences of carbamoyl phosphate synthetases from plants *Synechocystis* sp. and *Arabidopsis thaliana* (Figure 7). Although the biochemical and allosteric properties of the plant enzymes have not yet been characterized, the conservation of this constellation of amino acids in the regulatory region suggests that the chloroplast enzymes might also be regulated by UMP and IMP.

In constrast to the bacterial enzymes, mammalian pyrimidine-specific carbamoyl phosphate synthetases are activated by PRPP and inhibited by UTP. Given the chemical

similarities between PRPP and the phosphoribosyl moiety of IMP, the question of whether PRPP and UTP occupy the same or different sites in the homologous binding domain of the mammalian proteins is raised. Domain exchange (*9*) between *E. coli* carbamoyl phosphate synthetase and hamster CAD has shown that PRPP and UTP bind within the C-terminal domain of CAD. The mutation Asp1363Asn in hamster CAD (corresponding to Asn992 in *E. coli*) showed (*25*) that the mutant CAD became insensitive to PRPP. The results of these studies are consistent with the view that PRPP and UTP are also bound in topologically equivalent sites. The mechanism through which these regulatory molecules modulate carbamoyl phosphate synthesis will require crystallographic comparison of the native and complexed forms of the proteins.

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