1H NMR studies on the catalytic subunit of aspartate transcarbamoylase

tyrosine resonances/pK determinations/active-site ligand perturbations/mutant proteins

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ABSTRACT  The 1H NMR spectrum of the catalytic subunit of Escherichia coli aspartate transcarbamoylase (EC 2.1.3.2) was simplified by using strains auxotrophic for the aromatic amino acids and a growth medium containing fully deuterated Trp, Phe, and His and partially deuterated Tyr. 1H resonances for Tyr in the catalytic trimer (Mr = 105) were partially resolved into five peaks at 27°C, which above 50°C were further resolved to give a distinct resonance for each of the eight Tyr residues in the polypeptide chain. Experiments on chemically modified catalytic subunits and on a mutant form in which Tyr-165 was converted to Ser-165 led to the assignment of resonances for Tyr-165, Tyr-240, and Tyr-185. Binding of the substrate, carbamoyl phosphate, caused shifts of two of the unassigned resonances, and the subsequent binding of the aspartate analog succinate perturbed the resonances corresponding to Tyr-165 and Tyr-240. The bisubstrate analog N-(phosphonacetyl)-L-aspartate produced a spectrum differing considerably from that caused by the combination of carbamoyl phosphate and succinate. The NMR spectrum for the Tyr-165 → Ser mutant trimer showed clearly that the single amino acid substitution caused conformational changes affecting the environment of residues remote from the position of the replacement. In contrast, the inactive mutant subunit in which Gly-128 was replaced by Asp exhibited a spectrum virtually identical to that of the wild-type protein. However, addition of the substrate carbamoyl phosphate caused a marked change in the spectrum of the mutant enzyme, whereas that of the wild-type trimer was altered only slightly, showing that the effect of the amino acid substitution was manifested in the NMR spectrum only with the liganded enzyme.

Much of the research on the regulatory enzyme aspartate transcarbamoylase (ATCase; aspartase carbamoyltransferase, carbamoyl-phosphate: L-aspartate carbamoyltransferase, EC 2.1.3.2) from Escherichia coli has been focused on the mechanism of the allosteric transition whereby active-site ligands promote the conversion of the enzyme from a low-activity, compact, or tight (T), conformation to a more active, swollen, or relaxed (R), form (1–3). This effort has resulted in detailed knowledge of the structures of both the T and R conformations (4–8). By comparison, little is known about the detailed structure of the isolated catalytic (C) trimer and the conformational changes resulting from the binding of active-site ligands. Although the crystallization of the C subunit and preliminary diffraction data were reported 11 years ago (9), the x-ray diffraction studies have yet to yield information about the tertiary and quaternary structure of the trimeric protein. To what extent the folding of the chains, the interchain interactions, and the conformation at the active sites are altered when the C trimers combine with regulatory dimers to form the holoenzyme is not known. It is clear that the catalytic properties of the C trimer as well as its affinity for active-site ligands differ from those of both the T and R forms of intact ATCase (10–12). But these differences in functional properties cannot be interpreted because of the lack of a high-resolution structure for the C trimer. It seemed of interest, therefore, despite the relatively large size of the C trimer (Mr = 105), to initiate NMR studies in an attempt to determine the environment of those amino acid residues for which resonances can be assigned and to examine the perturbations resulting from the binding of active-site ligands. Although extensive NMR studies have been conducted previously on both the C subunit and intact ATCase (13–20), most of these investigations were focused not on the structure of the enzyme but on the mechanism of binding specific ligands. These studies exploited changes in the resonances of various nuclei in the low molecular weight compounds. Only in experiments with [19F]fluorotyrosine-labeled ATCase (19) and [13C]labeled C (20) and regulatory (18) subunits were the signals from the protein observed directly, and E. coli strains auxotrophic for Tyr or His were employed to produce ATCase enriched in either [19F]fluorotyrosine or [13C]His. This general approach with strains auxotrophic for aromatic amino acids was utilized in the work presented here. In addition, the 1H NMR spectrum in the aromatic region was greatly simplified by using completely deuterated Trp, Phe, and His and partially deuterated Tyr in the growth medium. By this means, high-resolution 1H NMR spectra of the Tyr in the C subunit were obtained, and it was possible to assign several resonances and determine the stability of the C trimer at high temperature. Experiments were conducted to monitor alterations of Tyr environments caused by different active-site ligands and amino acid substitutions in mutant forms of the C subunit.

EXPERIMENTAL PROCEDURES

Strains and Growth Media. The following E. coli strains were constructed for the production of wild-type and mutant forms of ATCase enriched with specifically deuterated aromatic amino acids: HS1052 (aroA- his- pyrF701/pPYRB3), HS1068 (aroA- his- pyrF701/pYPYRB3), and HS1076 (aroA-hisGΔ carAB- arg- pyrB-)/pPYRB766). The plasmids pPYRB3 and pPYRB766 carry a wild-type (21) allele of ATCase and a mutant allele leading to the replacement of Tyr-165 by Ser (22). pyrB231 is a chromosomal allele expressing a Gly-128 → Asp replacement (23). Lesions in

Abbreviations: ATCase, aspartate transcarbamoylase; C, catalytic; PALA, N-(phosphonomethyl)-L-aspartate; CbmP, carbamoyl phosphate.

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pyrimidine biosynthesis (pyrF701 and carAB−) facilitated high expression of ATCase from the pyrB-pyrI promoter, and auxotrophy for aromatic amino acids (aroA and his mutations) allowed for efficient incorporation of exogenously supplied Trp, Tyr, Phe, and His. Cells were grown in M6 minimal medium supplemented with uracil (4 mg/liter), p-aminobenzoic acid (12 μg/liter), L-[2,4,5,6,7-2H]Trp (7 mg/liter), L-[2,4,6-2H]Tyr (40 mg/liter), L-[2,3,4,5,6-2H]Phe (40 mg/liter), and DL-[α,2,4-2H]His (20 mg/liter); for strain HS1076, Arg at 100 mg/liter was included, and ampicillin (50 mg/liter) was added to growth media for strains containing the pPRB plasmids.

Deuterated Amino Acids. L-[2,4,5,6,7-2H]Trp and DL-[α,2,4-2H]His were prepared by acid-catalyzed exchange in \( \text{H}_2\text{O}/\text{H}_2\text{SO}_4 \) (25, 26), and the 89% ring-deuterated product was treated further with \( \text{H}_2\text{O} \) and Adam’s catalyst (27, 28) to yield \([2,3,4,5,6-2\text{H}]\text{Phe}\), of which ~90% remained as the L-isomer. L-[2,3,5,6-2H]Tyr was kindly provided by Tom Walker of Los Alamos National Laboratory. This material was converted to L-[2,6-2H]Tyr by mild acid-catalyzed exchange in \( \text{H}_2\text{O} \) (24). All deuterated amino acids were determined by \(^1\text{H} \) NMR spectroscopy to be >96% isotopically enriched, and the extent of racemization was evaluated from the amount of \(^1\text{H} \) exchange.

NMR Spectroscopy. Protein samples for NMR spectroscopy were 8–15 mg/ml and were exchanged into 99.8%-deuterated buffer solutions by dialysis and repeated cycles of dilution and concentration in Centricon-10 (Amicon) microconcentrators. For some samples, lyophilization and reconstitution with \( \text{H}_2\text{O} \) (99.8% \(^2\text{H} \)) was the final step. Unless otherwise specified, samples were in 40 mM potassium phosphate, 1 mM dithiothreitol, and 0.2 mM EDTA at pH 7.0 (pH meter readings are uncorrected for the deuterium isotope effect). Chemical shifts are referenced to internal sodium 2,2-dimethyl-2-silapentane-5-sulfonate (=0.05%). For pH titrations, 1 mM piperezine and 1 mM ethylenediamine were included as internal pH indicators. The \(^1\text{H} \) NMR chemical shifts of these compounds, determined as a function of pH in 40 mM potassium phosphate in \( \text{H}_2\text{O} \) at 27°C, were fit to two titration curves and yielded \( p\text{K}_\alpha = 7.73, p\text{K}_\beta = 10.34, \delta_{\text{acid}} = 3.368, \delta_{\text{intermediate}} = 2.995, \delta_{\text{base}} = 2.630 \) for ethylenediamine; and \( p\text{K}_\alpha = 5.84, p\text{K}_\beta = 10.19, \delta_{\text{acid}} = 3.583, \delta_{\text{intermediate}} = 3.116, \delta_{\text{base}} = 2.732 \) for piperezine. Proteins were titrated by addition of small amounts of KO\(^2\text{H} \) or \( \text{H}_2\text{PO}_4 \) directly to the NMR sample tube. From the ethylenediamine and piperezine chemical shifts, pH was determined by reference to calibration curves generated with the parameters listed above.

\(^1\text{H} \) NMR spectra were acquired at 500 MHz on a Nicolet NT-500 instrument at the University of California, Davis. A typical experiment employed quadrature detection with a spectral width of 7000 Hz, a selective 150-ms low power pulse to suppress the residual water resonance, and a 1.4-s total cycle time.

Other Methods. Wild-type and mutant ATCase and C subunits were isolated by procedures developed in this laboratory. Enrichment with deuterated amino acids had no discernible effect upon enzyme activities. C subunit was nitrated with tetranitromethane either as described by Kirschner and Schachman (29) or Lauritzen et al. (30). Whereas the first procedure yields a derivative nitrate predominantly at Tyr-240, both Tyr-165 and Tyr-240 are modified by the second method. The extent of modification was determined spectrophotometrically in 8 M urea at pH 8.0, assuming a nitrotyrosine extinction coefficient of 4100 M\(^{-1}\) cm\(^{-1}\) at 428 nm. C subunit concentrations were determined in phosphate buffer (40 mM potassium phosphate, pH 7.0/2 mM 2-mercaptoethanol/0.2 mM EDTA) by use of an extinction coefficient of 0.72 (mg/ml)\(^{-1}\) cm\(^{-1}\) at 280 nm.

Disodium N-(phosphonacetyl)-L-aspartate (PALA) was a gift from George R. Stark (Research Institute, Cleveland Clinic Foundation).

RESULTS

Resolution of the Tyr Residues in C Subunit. Dramatic simplification of the ATCase C subunit \(^1\text{H} \) NMR spectrum was achieved by means of incorporation of partially deuterated amino acids. Most resonances from the 124 aromatic protons were eliminated by the incorporation of fully ring-deuterated Phe, His, and Trp. Moreover, the use of L-[2,6-\(^2\text{H} \)]-Tyr reduced substantially the complexity of this region of the spectrum. Based on the equivalence of the three chains within each C subunit, only 16 aromatic \(^1\text{H} \) atoms remain in the partially deuterated protein. For those Tyr residues where rotation about the \( \text{C}^\text{α}–\text{C}^\text{γ} \) bond is rapid, magnetic equivalence of the protons at positions 3 and 5 on a given Tyr ring leads to a single resonance per Tyr. Thus, the aromatic region of the \(^1\text{H} \) NMR spectrum for the 100-kDa wild-type C subunit (C\( \text{wt} \)) can be reduced to between 8 and 16 Tyr resonances.

Fig. 1 shows that the Tyr resonances of C\( \text{wt} \) subunit at 50°C are partially resolved into five peaks. A similar spectrum is observed when the sample is maintained at 27°C in the standard phosphate buffer. Spectra obtained at progressively higher temperatures show a marked narrowing of the resonances, and at 70°C and 80°C, distinct peaks for all eight Tyr residues are apparent. Thus, at 50°C and below, the broad peaks at 6.3, 6.5, and 6.8 ppm each represent two overlapping resonances. The enhanced resolution achieved at higher...
normally performed by both Tyr subunit. Nitration of B. smegmatis obtained.

"loosening" temperatures is therefore, that the average conformation of the C<sub>wt</sub> subunit is essentially identical throughout the temperature range of the experiment and that the enhanced resolution at high temperature is a consequence of a general "loosening" of the structure to allow less restricted motion of the Tyr side chains. Above 80°C, irreversible aggregation of the protein occurred, and additional spectra could not be obtained. Differential scanning calorimetry of C<sub>wt</sub> trimer in the same buffer indicates a melting temperature of 84°C (C. B. Peterson and H. K. S., unpublished results), consistent with the stability observed in the NMR experiment.

Resonance Assignments for Tyr-165 and Tyr-240. Two approaches were utilized for the assignments of Tyr-165 and Tyr-240, both of which relied upon the deletion of specific Tyr resonances in a chemically or genetically modified C subunit. Nitration of the protein with tetranitromethane was performed by procedures known to modify Tyr-240 preferentially or both Tyr-165 and Tyr-240 (29, 30). Each 3-nitrotyrosyl residue thus formed would lack the 1H resonance(s) normally found between 6.2 and 6.9 ppm due to loss of one of the two ortho hydrogens and a downfield shift (31) of the other. Fig. 2A shows a spectrum of the [2,6-2H]<sub>Tyr</sub> residues of the native C<sub>wt</sub> subunit and the difference obtained upon subtraction of the spectrum of a derivative nitrated on both Tyr-165 and Tyr-240. Initial assignments of the difference peak at 6.81 ppm as the Tyr-165 resonance and the peak at 6.59 ppm as the Tyr-240 resonance were made by further studies of the protein nitrated primarily at Tyr-240. This modified protein contained only 0.4 nitrotyrosyl groups per polypeptide chain, and, when compared to the native C<sub>wt</sub> subunit, the difference spectrum revealed a major peak at 6.59 ppm and only a minor peak at 6.81 ppm. Confirmation of the assignments was achieved by the use of a mutant protein in which Tyr-165 was converted to Ser-165 by site-directed mutagenesis (22). A comparison of the Ser-165 C trimer (Cy<sub>165S</sub>) with the C<sub>wt</sub> subunit is shown in Fig. 2B. Spectra are shown for the proteins at pH 9.5 because, at this pH, the two resonances that underlie the 6.81 ppm peak (at pH 7.0) are shifted and resolved. The obvious absence of one resonance in the spectrum of the C<sub>Y165S</sub> subunit identifies it as arising from Tyr-165, thereby supporting the assignments indicated in Fig. 2A.

Tyrosine pK<sub>a</sub> Values and Assignment of the Tyr-185 Resonance. Spectra for the C<sub>wt</sub> subunit were determined as a function of pH, from which four pK<sub>a</sub> values could be fit to single-proton titration curves (Fig. 3). Within the range of pH 7–11, the titration behavior was completely reversible. The lowest Tyr pK<sub>a</sub>, 9.79, corresponds to Tyr-165 (see Fig. 2B), which is consistent with the relatively facile nitration of that residue. This simple correlation may be misleading, however, because Tyr-240 has a pK<sub>a</sub> of 10.94, which is substantially higher than the pK<sub>a</sub> values for two other, unassigned Tyr residues (pK<sub>a</sub> values of 10.19 and 10.27), which are not readily nitrated.

The excellent fits to single-proton ionizations within a pH range of 9–11 suggest that the titration curves in Fig. 3 represent Tyr pK<sub>a</sub> values and are not secondary effects due to titrations of other amino acid residues. An example of the latter situation was found upon adjusting the sample to pH 6.45. At this lower pH, the broad peak at 6.48 ppm split into two resonances at 6.47 and 6.39 ppm, an effect that is probably attributable to protonation of a His side chain. Inspection of the ATCase crystal structure (4, 6) revealed that only one Tyr, Tyr-185, is in close proximity to a His residue. In fact, Tyr-185 has two neighboring His residues. With the assumption that the low pH effect is due to a direct influence of the protonation of His-156 and/or His-212, the perturbed resonance can be assigned to Tyr-185.
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Effects of Active-Site Ligands. As seen in Fig. 4, the addition of the substrate carbamoyl phosphate (CbmP) has a slight but significant effect on the Tyr \(^1\)H NMR spectrum. The broad resonances at 6.25 and 6.48 ppm, representative of two unassigned Tyr residues, are shifted as a result of the binding. Upon the subsequent addition of succinate, an analog of the substrate aspartate, the resonances corresponding to Tyr-165 and Tyr-240 are perturbed. The NMR spectrum was altered further upon the addition of the bisubstrate analog PALA, which binds to the C trimer with high affinity (11, 32) and probably displaces the CbmP and succinate at the active sites. It is clear that PALA causes a broadening and slight downward shift of the Tyr-165 resonance [assigned by comparison with the spectrum for the CbmP subunit plus ligands] and a major upfield shift (0.05 ppm) of the Tyr-240 resonance.

Effects of Amino Acid Substitutions on the NMR Spectrum of C Subunit. Fig. 5A shows the marked change in the Tyr \(^1\)H NMR spectrum of the C subunit as a result of the substitution of Ser for Tyr-165. In addition to the expected loss of half the intensity of the 6.8 ppm peak, the amino acid substitution results in coalescence of the 6.65 ppm (unassigned) and 6.60 ppm (Tyr-240) resonances. This comparison of the spectrum of Cwt subunit and that of the mutant trimer indicates convincingly that a single amino acid substitution can cause conformational changes remote from the position of the replacement. The distance between Tyr-165 and Tyr-240, deduced from the crystal structure of wild-type holoenzyme (6), is about 22 Å.

A comparison between the Cwt trimer and a mutant protein containing a Gly-128 \(\rightarrow\) Asp replacement (C128D) is illustrated in Fig. 5B. The \(^1\)H NMR spectra of the unliganded C trimers are virtually identical. However, the two C trimers in the presence of CbmP exhibit markedly different \(^1\)H NMR spectra. Thus the manifestation of the amino acid substitution is revealed only after the binding of the substrate.

DISCUSSION

Sufficient quantities of the C subunit of ATCase for high-resolution \(^1\)H NMR spectroscopy were obtained by using \(E. coli\) strains auxotrophic for aromatic amino acids and adding fully ring-deuterated Phe, His, and Trp along with partially deuterated Tyr during the growth of the bacteria. In this way, most of the resonances in the aromatic region were eliminated and only those of the partially deuterated Tyr resonances were detected. At room temperature the C subunit, consisting of three identical 33-kDa polypeptide chains, exhibited a spectrum composed of five partially resolved peaks. Heating the preparation of the C trimer yielded spectra (Fig. 1) with a marked narrowing and splitting of some of the peaks, resulting at 70°C–80°C in eight distinct resonances corresponding to the eight Tyr residues per chain. It should be noted that the unusual heat stability exhibited by the C trimer in the NMR spectra was confirmed by differential scanning calorimetry experiments on the protein in the same buffer. The melting temperature of 84°C, which is significantly higher than previously reported (33), is attributable to the presence of \(1 \text{M} \text{Na}_2\text{SO}_4\).

A combination of chemical modification and site-directed mutagenesis permitted assignment of resonances of Tyr-165 and Tyr-240 as shown in Fig. 2A. An additional assignment of the resonance of Tyr-185 was made based on the measurements of the pKa values from titration curves (Fig. 3) and the \(^1\)H NMR spectrum at pH 6.45 in which the broad peak at 6.48 ppm is split into two resonances. This effect probably results from the protonation of a neighboring His side chain, and only Tyr-185 is located near His residues (6).

The spectra in Fig. 4 show clearly that some of the unassigned resonances are perturbed specifically as a result of CbmP binding. More extensive changes, affecting for
example the resonances corresponding to Tyr-165 and Tyr-240, occur upon the subsequent binding of succinate. Because catalysis by wild-type ATCase proceeds largely by ordered binding of CbmP followed by aspartate, it is important that models of the catalytic mechanism be based on the CbmP-ligated enzyme. Hence, it is necessary to know how the structure of unliganded ATCase is altered upon binding CbmP. Regrettably, the perturbations in some of the resonances described here are not yet interpretable in terms of actual structural alterations. In this regard, the differences in the spectra for the C subunit in the presence of CbmP and succinate, on the one hand, and in the presence of PALA, on the other, show clearly the hazards in using the structure of the enzyme liganded with the bisubstrate analog for interpretations of the catalytic mechanism.

Replacing Tyr-165 by Ser, as seen in Fig. 5A, has a greater effect than that expected from the simple elimination of a single resonance. Not only is there the expected decrease (about 50%) in the intensity at 6.8 ppm because of the amino acid substitution, but also there are marked alterations in other regions of the spectrum. The changes in the Tyr-240 resonance at 6.65 ppm and the resonance at 6.65 ppm from an as yet unidentified Tyr can be interpreted only by postulating a "global" conformational change in the C trimer affecting residues remote from the site of the amino acid replacement. According to the x-ray diffraction studies (6), the distance between Tyr-165 and Tyr-240 is >20 Å. Hence the effects of the substitution of Ser for Tyr-165 are propagated a considerable distance.

Of the many mutant forms of ATCase that have been examined thoroughly, that involving the replacement of Gly-128 by Asp (23) in the catalytic chains is the most perplexing. Its enzyme activity is about 10⁻³ (or less) that of wild-type ATCase, its heat stability is greatly decreased, the stability of the mutant holoenzyme toward subunit exchange is diminished, the interchain interactions with the C trimers are strengthened, and the chemical reactivities of the single Cys residue and the active site Lys-84 are substantially different from those same residues in the wild-type enzyme (23). Nonetheless, as seen in Fig. 5B, the 1H NMR spectrum of the C subunit containing the Gly-128 → Asp replacement is virtually identical to that of the wild-type C trimer. Only upon the addition of CbmP are the differences between the wild-type and mutant C trimers revealed by the striking variations in the NMR spectra. Clearly an understanding of the loss in activity and the ability to bind succinate will depend upon determining the structure of the CbmP-ligated C subunit as well as the structure of the unliganded C trimer.

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