FAD, 68385-36-4; 7-chloro-8-demethyl-FAD, 76510-45-7; D_2, 7782-39-0.

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Comparison of Aspartate Transcarbamoylases from Wheat Germ and *Escherichia coli*: Functionally Identical Histidines in Nonhomologous Local Sequences†

Stephen C. J. Cole and Robert J. Yon*

School of Biological Sciences and Environmental Health, Thames Polytechnic, London SE18 6PF, U.K.

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ABSTRACT: Aspartate transcarbamoylase (ATCase) from wheat germ and the catalytic subunit of the enzyme from *Escherichia coli* are trimers of similar size. The former is a regulatory enzyme in its trimeric state, while the latter is a component of a complex regulatory dodecamer. In a comparison of the two enzymes, reaction with diethyl pyrocarbonate revealed a highly active, essential histidine residue in each case. The two histidines (i.e., one in each enzyme) behaved nearly identically with respect to the following functional properties: (1) kinetics of acylation (ethoxyformylation) and concomitant inactivation; (2) kinetics of deacylation by hydroxylamine and concomitant reactivation; (3) hyperbolic dependence of the apparent first-order rate constant (k_{app}) on diethyl pyrocarbonate concentration; (4) pH dependence of k_{app}; (5) failure of active-center ligands to protect the residue against diethyl pyrocarbonate, producing instead near-identical increases in the inactivation rate. These similarities point to an essential, highly conserved histidine in each enzyme, in a functional microenvironment that has changed relatively little since the divergence of plants and bacteria. Ethoxyformylated peptides were isolated from tryptic digests of the two inactivated enzymes. Sequencing of the major labeled peptide in each case showed the wheat and *E. coli* histidines embedded in nonhomologous primary segments, suggesting that, contrary to expectation, these segments are not part of the conserved microenvironment. In the case of the *E. coli* enzyme, the essential residue was identified as His-134 in the known sequence, which has a potential catalytic role on crystallographic evidence [Krause, K. L., Volz, K. W., & Lipscomb, W. N. (1985) *Proc. Natl. Acad. Sci. U.S.A. 82*, 1643–1647]. A second, much less reactive histidine was identified as His-64. Since the full primary and tertiary structures of the wheat-germ enzyme are not known, it is not possible at present to compare the environments of the essential histidine in the two enzymes.

Aspartate transcarbamoylase (EC 2.1.3.2) catalyzes an early step in a major growth pathway, the de novo synthesis of pyrimidines. It exhibits great phylogenetic diversity in structural and regulatory properties. Structural types vary from relatively small (ca. 100 kDa) enzymes with few (3–4) chains in some bacteria and plants, through the larger (ca. 300 kDa) and more complex dodecameric structure of *Escherichia coli* and other enterobacteria, to very large complexes with

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* Author to whom correspondence should be addressed.

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multifunctional polypeptides that include other pyrimidine-pathway enzymes in fungi and animals [see Yon (1982) for literature citations]. Several of these enzymes are trimers or include trimeric sub- or superstructures; the trimeric state is relatively rare among proteins in general but common among carbamoyltransferases (Vickers, 1981).

We are engaged in comparative structure–function studies of two enzymes with similar quaternary structures but very different regulatory characteristics. Aspartate transcarbamoylase from wheat and the catalytic subunit of the complex enzyme from *E. coli* are trimers of 36- and 33-kDa chains, respectively (Yon et al., 1982; Weber, 1968). In the allosteric *E. coli* holoenzyme, the catalytic trimer is part of a complex dodecameric structure which also includes a dimeric regulatory subunit (Weber, 1968; Wiley & Lipscomb, 1968). In contrast, the wheat-germ trimer is a fully allosteric enzyme in its own right (Yon, 1972, 1984). Despite this difference in regulatory behavior, the two enzymes have similar kinetic mechanisms, and the stereospecificity of the L-aspartate binding site is essentially the same (Davis et al., 1970; Grayson et al., 1979). Inactivation studies with phenylglyoxal suggested an arginine residue with similar properties in the carbamoyl phosphate binding sites of the two enzymes (Kantrowitz & Lipscomb, 1976; Cole et al., 1986). In the *E. coli* enzyme, several other residues with potential active-center functions have been identified by chemical reactivity experiments, by site-specific mutagenesis, and by protein crystallography [summarized in Krause et al. (1985)]. Among these, one or more histidine residues have been implicated. Photooxidation of two histidines was mediated by a pyridoxylated lysine that was shown to be in the active center (Greenwell et al., 1973; Kempe & Stark, 1975). Recently, His-134 has been located near the active center by crystallography, and a catalytic role for this histidine has been suggested (Krause et al., 1985). However, the use of the relatively histidine-specific reagent diethyl pyrocarbonate (Miles, 1977) has not previously been reported in relation to aspartate transcarbamoylase. In the present paper we describe comparative experiments with this reagent that suggest a conserved, highly reactive histidine in the two trimeric aspartate transcarbamoylases.

Experimental Procedures

Materials. The purified catalytic subunit of ATCase[1] from *E. coli* was a gift from Dr. Guy Hervé, CNRS, Gif-sur-Yvette, France. Wheat-germ ATCase was purified as described previously (Yon, 1981), except that the two ethanol precipitation steps were combined. PALA was a gift from Dr. George Stark, Imperial Cancer Research Fund Laboratories, London, U.K.

Assay. Wheat-germ ATCase was assayed as described by Yon (1972). The *E. coli* catalytic subunit was assayed in 0.1 M Tris–acetate buffer, pH 8.5, with L-aspartate at 10 mM and carbamoyl phosphate at 5 mM; otherwise the assays were identical.

Molar enzyme concentrations were calculated from A_{280} measurements and molecular weights of 100 kDa (*E. coli* catalytic subunit) and 104 kDa (wheat-germ enzyme). For the catalytic subunit the extinction coefficient at 280 nm was taken to be 0.71 $mL \cdot mg^{-1} \cdot cm^{-1}$ (Gerhart & Schachman, 1965). The extinction coefficient for the wheat enzyme was the average of three determinations, each involving a weight determination by amino acid analysis of an aliquot of known absorbance at 280 nm. The extinction coefficient was found to be 1.12 $mL \cdot mg^{-1} \cdot cm^{-1}$.

Inactivation by Diethyl Pyrocarbonate. The standard procedure for inactivating the enzyme was as follows. The enzyme (1 μM) was dissolved in appropriate buffer at 25 °C in a 1-cm quartz cuvette. A concentrated stock solution of diethyl pyrocarbonate was added to give the required final concentration. The reaction was monitored at 242 nm in a Perkin-Elmer Model Lambda 3 spectrophotometer connected to a recorder giving full-scale deflection at an absorbance of 0.05. Small aliquots were removed at intervals for enzyme assay; assay conditions were chosen to ensure adequate quenching of the acylation reaction. When varied, buffer conditions and reagent concentrations are described for each experiment. For constant-pH experiments, the buffer was 0.1 M sodium bicarbonate, pH 8.5. For most experiments, an initial concentration of 50 μM diethyl pyrocarbonate was used.

The stock solution of diethyl pyrocarbonate was made up in ethanol and stored at −20 °C for up to 2 days prior to use. Its concentration was determined by reaction to completion with a large excess of *N*-acetylhistidine (Holbrook & Ingram, 1973). For quantitative determination of reacted histidine, the extinction coefficient of monoethoxyformylhistidine was taken as 3600 $M^{-1} \cdot cm^{-1}$ (Holbrook & Ingram, 1973).

Isolation of Labeled Peptides. Three milligrams of *E. coli* catalytic subunit or 0.5 mg of wheat-germ enzyme in 0.1 M sodium bicarbonate buffer, pH 8.5, was reacted with 3 mM diethyl pyrocarbonate in a quartz cuvette until the absorbance at 242 nm indicated the formation of 1 ethoxyformylhistidine residue per chain (about 10 min). The solution was then filtered through a column of Sephadex G10 (22 × 0.8 cm) in 50 mM ammonium bicarbonate, pH 7, to remove excess diethyl pyrocarbonate and to stop the reaction. Trypsin was then added to the ethoxyformylated enzyme in a weight ratio of 1:100 and the mixture incubated at 30 °C for 8 h. The mixture of tryptic peptides was chromatographed on a column of Sephadex G15 (60 × 1.5 cm) in 50 mM ammonium bicarbonate, pH 7. Fractions containing ethoxyformylated peptides (revealed by their absorbance at 242 nm) were freeze-dried. Their purity was examined, and, if necessary, they were further purified by high-voltage electrophoresis on Whatman 3MM paper (58 × 28 cm) in acetic/formic acid buffer, pH 1.9. At this low pH the ethoxyformyl label was lost; thereafter histidine was identified in peptides by the Pauly spray reagent (Perham, 1978). Prior to sequencing, the purity of each peptide was confirmed by electrophoresis at a different pH (usually pyridine/acetic acid, pH 6.5). Peptides were eluted from the paper into 50 mM ammonia and concentrated by freeze-drying. Portions of the purified tryptic peptides were further digested with elastase and the resulting peptides again purified by electrophoresis.

Amino Acid Sequence Analysis. The N-terminal sequences of the various peptides were determined by the indirect dansyl–Edman technique. The Edman degradation was performed as described by Perham (1978). Dansylation of the peptides and standard amino acids was performed as described by Weiner et al. (1972). Dansyl amino acids were separated and identified by thin-layer chromatography on double-sided polyamide plates (Gray, 1972).

Results

Preliminary Experiments. The majority of published reports of histidine modification by diethyl pyrocarbonate indicate that phosphate at pH 7 is the preferred buffer system. We wished to avoid this compound, which is a ligand of the

[1] Abbreviations: ATCase, aspartate transcarbamoylase; PALA, *N*-(phosphonoacetyl)-L-aspartate; Tris, 2-amino-2-(hydroxymethyl)-1,3-propanediol; UMP, uridine 5′-monophosphate; CTP, cytidine 5′-triphosphate.

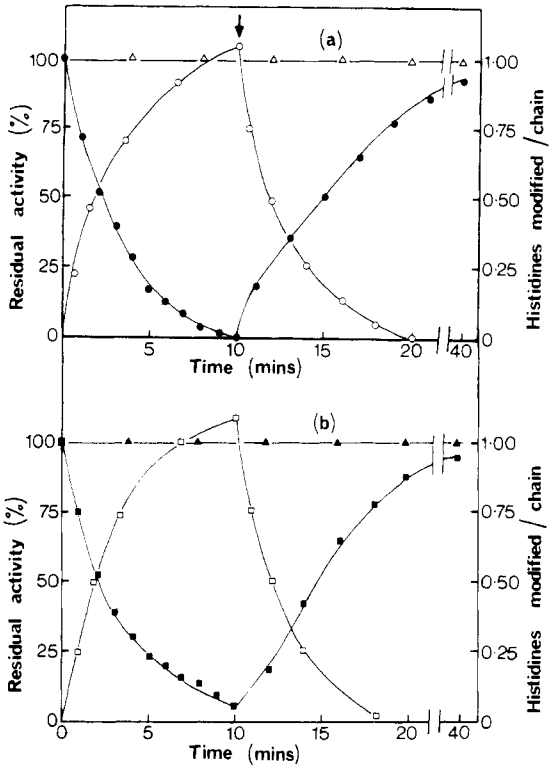

FIGURE 1: Inactivation and acylation of wheat-germ and *E. coli* ATCases by diethyl pyrocarbonate. Change of enzyme activity (●, ■) and histidines modified per chain (○, □) as functions of time for (a) wheat-germ and (b) *E. coli* ATCases. Activity is shown in the presence (●, ■) and absence (△, ▲) of 50 μM diethyl pyrocarbonate and is expressed as a percentage of the initial activity. After 10 min (arrow), 0.5 M hydroxylamine was introduced.

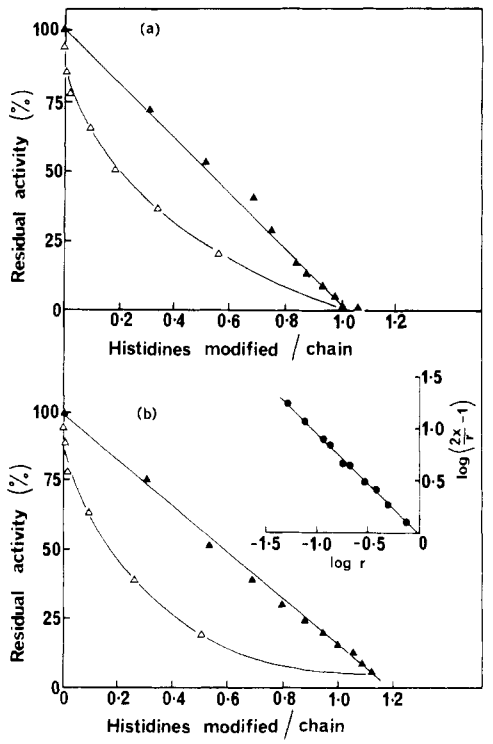

FIGURE 2: Correlation of residual activity and histidine modification. Relation between residual activity and the number of histidines modified before (▲) and after (△) the introduction of hydroxylamine for (a) wheat-germ and (b) *E. coli* ATCases. (Inset) Tsou plot (Tsou, 1962) assuming two modifiable histidines of which one (the more rapidly reacting) is essential: x = fraction of modifiable histidines unreacted and r = residual activity (A/A_0).

enzyme, and to use a higher pH. The present experiments were therefore performed in bicarbonate buffer, pH 8.5. Diethyl pyrocarbonate (50 μM) was found to have a half-life of 15.5 min at pH 8.5 in this buffer; therefore, the reagent was in excess (at least 10-fold) over active sites for the first 10 min. Greater excess was avoided to minimize the formation of diethoxyformylhistidine (Miles, 1977). There was no change in A_{280} in this period, indicating no significant reaction by tyrosines (Melchior & Fahrney, 1970). Thiols have been shown to react with diethyl pyrocarbonate in carboxylate buffers, the products having significant A_{242} (Garrison & Himes, 1975). However, we found no evidence of this in the bicarbonate buffer system used here when 2-mercaptoethanol was used as a model compound. We conclude that cysteines are not responsible for the inactivation reported below.

Inactivation and Ethoxyformylation Reactions. Figures 1 and 2 show that both enzymes (1 μM) are rapidly inactivated by diethyl pyrocarbonate (50 μM), with a half-time of about 2 min. Concomitantly, a rise in A_{242} indicates the acylation (ethoxyformylation) of histidine. Loss of activity coincides in time with the modification of 1.0 (wheat enzyme) and 1.2 (*E. coli* enzyme) histidines per chain. In both instances, reaction for longer periods (up to 1 h) resulted in the modification of no more than 2 histidines in all. The *E. coli* data were analyzed by the method of Tsou (1962) [see also Horiike and McCormick, (1979)]. An excellent straight line, with the predicted intercepts, was obtained on the assumption of two reacting histidines of which one is essential (Figure 2b, inset). The analysis indicates that the nonessential histidine reacts at about one-twentieth the rate of the essential histidine. This is in agreement with the finding of two modified histidines with different reactivities in structural studies on the *E. coli* catalytic subunit (see below).

Reactivation and Deacylation Reactions. Figures 1 and 2 also show that, in both enzymes, the introduction of 0.5 M hydroxylamine after 10 min resulted in a rapid deacylation (fall in A_{242}) accompanied by recovery of activity to about 95% of the initial activity. This behavior is reported to confirm that the essential residues modified were histidines, since the deacylation of other modified residues proceeds much more slowly (Burstein et al., 1974). Moreover, the near-quantitative recovery of activity indicates that, if other residues are modified by diethyl pyrocarbonate, they are not essential for activity. Unlike the coincidence of inactivation and acylation, however, the reactivation lags behind deacylation (Figure 2, △). This may indicate some conformational change following the acylation reaction.

Effect of Diethyl Pyrocarbonate Concentration on Inactivation Rate. When the inactivation stages in Figures 1 and 2 were plotted as semilogarithmic (first-order) time plots, they were linear for the whole 10 min. This was surprising, since the reagent was known to decompose fairly rapidly at pH 8.5, and the inactivation rate might be expected to decrease with reagent concentration. Accordingly, the dependence of the apparent first-order inactivation rate constant (k_{app}, the slope of the semilogarithmic time plot) on reagent concentration was investigated with the results shown in Figure 3. Surprisingly, instead of the linear dependence expected, both enzymes showed a hyperbolic dependence of k_{app} on reagent concentration, with similar half-saturation concentrations of about 5 μM. This result shows that the enzyme binds the reagent noncovalently, the acylation reaction proceeding within this enzyme–reagent complex. This explains why, providing the residual concentration is sufficient to saturate the enzyme (i.e., is greater than about 30 μM), the inactivation rate is not affected by the decomposition of the reagent.

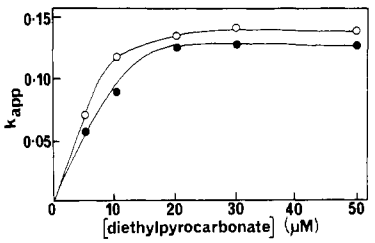

FIGURE 3: Effect of diethyl pyrocarbonate concentration on inactivation rate. The effect of reagent concentration on the apparent first-order inactivation rate constant is shown for (○) wheat-germ and (●) *E. coli* ATCases.

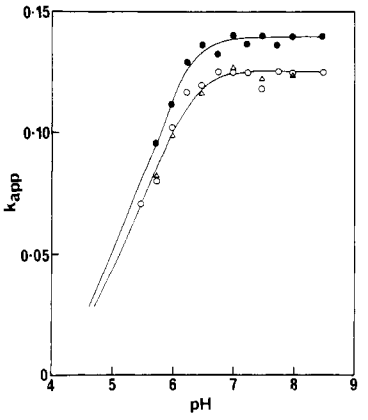

FIGURE 4: pH dependence of the apparent first-order inactivation rate constant. The effect of pH is shown for the inactivation by diethyl pyrocarbonate of (●) wheat-germ and (○) *E. coli* ATCases. Open triangles show the effect of pH on the rate of ethoxyformylation. Buffer compositions were, pH 5–7, 0.1 M citric acid/trisodium citrate and, pH 7–8.5, 0.1 M $NaHCO_3/Na_2CO_3$. The lines are theoretical curves, calculated from $k_{app} = k_{max}/(1 + [H^+]/K_a)$, where k_{max} is the plateau value of k_{app} and it is assumed that k_{app} falls to zero at low pH.

pH Dependence of Inactivation Rate Constant (k_{app}). The gradients of semilogarithmic time plots of the inactivation (i.e., k_{app}) were examined at pH values between 5.7 and 8.5, with the reagent concentration held at 50 μM. In both enzymes, catalytic activity was found to be unstable below pH 5.7. The results are shown in Figure 4. In the case of the *E. coli* enzyme the pH dependence of the rate constant for ethoxyformylation was also examined (Figure 4, △); the curves for ethoxyformylation and for inactivation coincided exactly. Insufficient protein precluded this experiment for wheat-germ ATCase.

For both enzymes the k_{app} vs. pH curves suggest a pK_a value of about 5.4. Since the interaction of diethyl pyrocarbonate with the enzyme involves binding residues in addition to the reactive histidine (see previous paragraph), it is not possible on present evidence to assign this pKa to the reactive histidine unambiguously.

Effects of Active-Center Ligands on Inactivation Rate. In both the wheat and *E. coli* enzymes, the two substrates bind in an obligatory order, carbamoyl phosphate binding first (Porter et al., 1969; Grayson et al., 1979). The effects of carbamoyl phosphate alone, carbamoyl phosphate plus succinate (an analogue of the second substrate, L-aspartate), and the bisubstrate analogue PALA on the inactivation rate were studied. Surprisingly, none of the active-center ligands protected either enzyme against diethyl pyrocarbonate. Instead, in every case the enzyme became more susceptible to inactivation in the presence of the ligand. The effects were remarkably similar in the two enzymes. For wheat-germ ATCase the relative increases in the apparent first-order rate

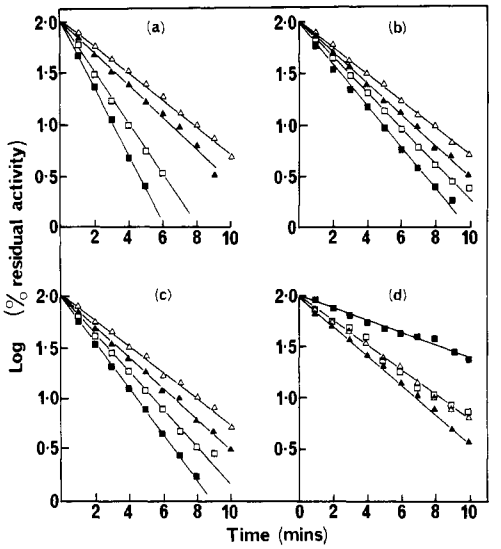

FIGURE 5: Effects of ligands on enzyme inactivation by diethyl pyrocarbonate. Residual activity data are plotted as linear first-order plots for the wheat-germ enzyme (▲, ■) and the *E. coli* catalytic subunit (△, □). In each case the effect of a saturating concentration of ligand (■, □) is compared with control plots (▲, △). The ligands tested were (a) carbamoyl phosphate, (b) carbamoyl phosphate plus succinate, (c) PALA, and (d) UMP.

constant (compared to the unliganded enzyme) were: 2.1-fold for carbamoyl phosphate; 1.3-fold for carbamoyl phosphate plus succinate; 1.4-fold for PALA. The corresponding increases for the *E. coli* catalytic subunit were 2.0-fold for carbamoyl phosphate, 1.4-fold for carbamoyl phosphate plus succinate, and 1.4-fold for PALA.

Effects of UMP and CTP on Inactivation Rate. UMP is a potent feedback inhibitor of wheat-germ ATCase (Yon, 1972, 1984) but has no regulatory effect on the *E. coli* enzyme. The effect of UMP on the reactive histidine agrees with this distinction; a partial protection of the wheat enzyme only is seen when UMP is used at a concentration (100 μM) that would saturate the wheat enzyme. It has been suggested that the catalytic subunit of the *E. coli* enzyme is able to bind CTP (Issaly et al., 1982); however, this compound at 5 mM had no effect on the inactivation rate of either enzyme.

Isolation and Sequencing of Labeled Peptides. (1) Catalytic Subunit of E. coli ATCase. Reaction with diethyl pyrocarbonate proceeded to the extent of 1.3 residues per chain. Figure 6a shows the elution profile of the tryptic peptides obtained from the labeled enzyme. There were two prominent ethoxyformylated peptide peaks indicating the most reactive and second most reactive histidines, respectively. The larger peak was pure by high-voltage electrophoresis; this peptide is designated ET1. An aliquot was set aside for sequencing, and the remainder was subjected to elastase digestion. From this digest a single histidine-containing peptide, EE1, and one other peptide, EE2, were purified by electrophoresis at pH 1.9 and subjected to N-terminal sequencing. From the smaller chromatographic peak (Figure 6a) a single histidine-containing peptide was purified by high-voltage paper electrophoresis and partially sequenced. Figure 7 shows N-terminal sequences of ET1 and its subfragments EE1 and EE2 aligned with part of the published sequence of the *E. coli* catalytic subunit (Hoover et al., 1983), including the putative peptide between the consecutive tryptic cleavage sites Arg-113 and Arg-151. This is the largest of the tryptic peptides that contains histidine. The first seven amino acids of peptide ET1 corresponded to the sequence Leu-114 to Gly-120, suggesting that ET1 was the large tryptic peptide. Hence the labeled histidine should

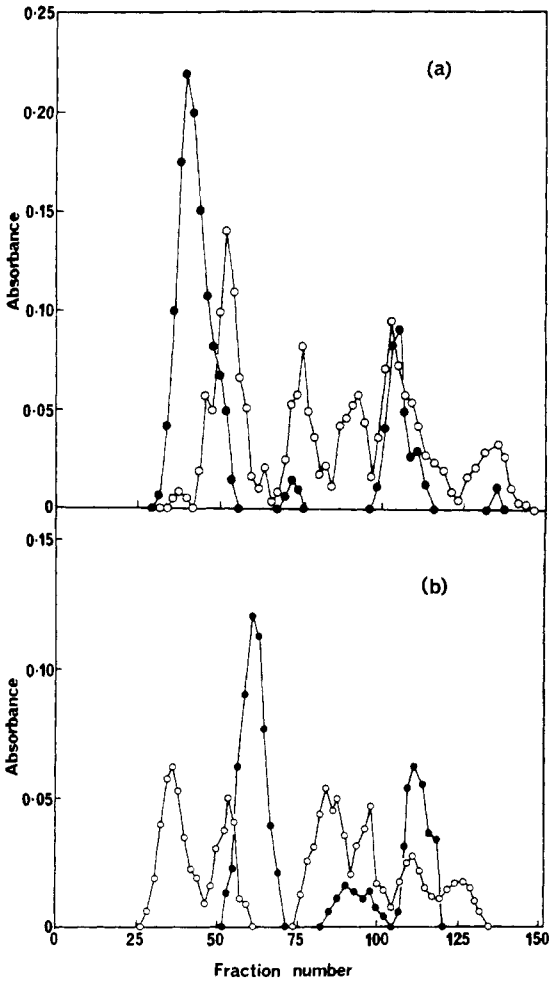

FIGURE 6: Gel filtration chromatography of ethoxyformylated tryptic peptides. Elution profiles are shown for liberated peptides from (a) *E. coli* and (b) wheat-germ ATCases. Open circles indicate A_{280}, and closed circles indicate A_{242}.

(a)

111 125
ala-ala-arg☆-leu-ala-thr-glu-phe-ser-gly-asn-val-pro-val-leu-
leu-ala-thr-glx-phe-ser-gly-
ET1

126 140
asn-ala-gly-asp-gly-ser-asn-gln-HIS-pro-thr-gln-thr-leu-leu-
gly-asp-gly-ser-asx-glx-HIS-pro-(thr,glx,leu)
EE1

141 155
asp-leu-phe-thr-ile-gln-glu-thr-gln-gly-arg☆-leu-asp-asn-leu-
glx-glx-thr-glx-gly
EE2

(b)

51 65
ala-ser-ile-arg☆-thr-arg☆-leu-ser-phe-gln-thr-ser-met-HIS-arg☆-
leu-ser-phe-glx-(thr,ser,met,HIS,arg)
ET2

FIGURE 7: N-Terminal sequences of ethoxyformylated peptides from *E. coli* ATCase: (a) peptide ET1 and elastase subfragments; (b) peptide ET2. In each case the numbered chain is the homologous portion of the catalytic subunit sequence (Hoover et al., 1983). Tryptic cleavage points are indicated (☆).

be His-134, the only histidine in the peptide. This was confirmed by the first eight residues of the elastase subfragment EE1 which corresponded to the sequence Gly-128 to Pro-135.

(a)

phe-thr-gly-asx-leu-HIS-ile-(asx,ser,gly,glx,ala,lys)
WT1

gly-asx-leu-HIS-ile-asx-ser-gly
WE1

glx-glx-gly-lys
WE2

(b)

129 143
asp-gly-ser-asn-gln-HIS-pro-thr-gln-thr-leu-leu-asp-leu-phe

phe-thr-gly-asx-leu-HIS-ile-asx-ser-gly-ala-glx-glx-gly-lys

FIGURE 8: Sequences of the ethoxyformylated tryptic peptide from wheat-germ ATCase: (a) peptide WT1 and elastase subfragments; (b) comparison with a portion of the *E. coli* sequence containing the labeled histidine [His-134 of the Hoover et al. (1983) sequence].

Peptide EE2 was found to be a pentapeptide corresponding to the sequence Gln-146 to Gly-150. Assignments are ambiguous only to the extent that Glu/Asp have not been distinguished from Gln/Asn. Aside from this, ET1 clearly corresponds to the tryptic peptide from Leu-114 to Arg-151, and the first-reacting histidine must be His-134.

The other labeled peptide ET2 was found to have the N-terminal sequence and amino acid composition of the tryptic peptide from Leu-57 to Arg-65, as shown also in Figure 2. It is therefore concluded that His-64 was the second most reactive histidine.

(*2*) *Wheat-Germ ATCase.* Figure 6b shows the elution profile of the tryptic peptides from wheat-germ ATCase. Again there were predominantly 2-ethoxyformylated peptide peaks. The major peak was found to contain a single peptide (WT1). Half of this purified peptide was subjected to sequencing and the remainder to elastase digestion as before. Electrophoresis of this digest indicated six components, one of which, WE1, contained histidine. This and one other elastase fragment (WE2) were partially sequenced. The secondary peak in Figure 6b was not characterized.

Figure 8 shows the sequences of the wheat-germ ATCase peptides WT1, WE1, and WE2. The first seven residues of WT1 included the histidine. There was clearly a five-residue overlap between WT1 and WE1, thus the first ten residues of WT1 were assigned unambiguously. Peptide WE2 was a tetrapeptide ending in lysine and was therefore the C-terminus of WT1. From the qualitative analysis of the residues remaining in WT1 after seven rounds of sequencing, the only residue not accounted for in WE1 and WE2 is alanine. WT1 is therefore tentatively denoted as the 15-residue peptide shown in the lower line of Figure 8b, with alanine in position 11.

When the histidines are aligned there is no sequence homology between WT1 and the sequence around His-134 of the catalytic subunit (Figure 8b), even when insertions/deletions are attempted. Likewise, we could find no homology with sequences close to any of the other 10 histidines in the *E. coli* sequence.

Discussion

(*1*) *General Comments.* From the known primary sequence, the catalytic subunit of the *E. coli* ATCase contains 11 histidines per chain (Hoover et al., 1983). Amino acid analysis of wheat-germ ATCase (S. C. J. Cole and R. J. Yon, unpublished work) suggests that this enzyme has eight histidines per chain. The experiments reported in this paper showed that in each case, one histidine was highly reactive compared to

the others, and that this histidine was essential for enzyme function. This function could in principle concern either the binding or catalytic phases.

It was somewhat disconcerting to find that no active-center ligand was able to protect either enzyme against reaction by diethyl pyrocarbonate. It is unusual, but not unprecedented, for ligand binding to increase, rather than decrease, the exposure of a residue to a modifying reagent. Such observations have been taken as strong evidence of a local conformation change induced by ligand binding [reviewed by Citri (1973)]. In both enzymes, carbamoyl phosphate (the first-binding substrate) most strongly increased the reaction rate; compared to this ligand, the effects of succinate or PALA (an analogue of the two substrates) were protective; i.e., the rates were lower (although still greater than that for the unliganded enzyme). A plausible explanation of these results is the following. The binding of carbamoyl phosphate is accompanied by a conformational change that increases the exposure of the essential histidine to diethyl pyrocarbonate. The subsequent binding of aspartate or succinate reduces this exposure, either by occlusion or by a further change in conformation. It is noteworthy that a catalytic mechanism for the *E. coli* catalytic subunit proposed by Collins and Stark (1969) was based on just such conformational changes and invoked a deprotonated general base that could well behave as postulated above for the essential histidine [see Figure 4 of Collins and Stark (1969)].

(*2*) *His-134 of E. coli ATCase.* Our finding that the essential histidine of the *E. coli* catalytic subunit is His-134 has relevance to recent X-ray diffraction studies of the *E. coli* enzyme. These report that His-134 is located close to the binding site for phosphates in the T-conformation (Ke et al., 1984) and to the carbonyl group of the bound bisubstrate analogue, PALA, in the R-conformation (Krause et al., 1985) of the regulatory holoenzyme. The latter authors state: "In our structure His-134, near the carbonyl group of PALA, is the only potentially catalytic side chain in this region, assuming that Ser-52 and Ser-80 are involved only in binding". The present results provide noncrystallographic evidence of a functional role for His-134, although whether in catalysis or in binding is less clear.

For reasons given in the Results section, the apparent pK_a of 5.4 in Figure 4 cannot at present be assigned to His-134 unambiguously. Such assignment would require evidence that the enzyme is saturated by diethyl pyrocarbonate at each pH value in Figure 4. Nevertheless it is worth commenting that such an apparently low pK_a would not be unrealistic for His-134 for two reasons. First, a potentiometric study (Allewell et al., 1979) identified (but did not assign) a pK_a of 5.5 in the catalytic subunit perturbed upon ligand binding. Second, an inspection of the three-dimensional environment shows that His-134 is located very close to the phosphate-binding site with Arg-54, Arg-105, and Arg-167 all within close distance (Krause et al., 1985). This positive "atmosphere" should favor the release of the histidine proton, accounting for the depressed pK_a value.

The catalytic mechanism of Collins and Stark (1969) invoked both general-acid and general-base groups in the carbamoyl-transfer reaction. His-134 is a candidate for either of these. We favor the role of the general base, for reasons given under General Comments above, and because it best suits a low pK_a for the residue.

(*3*) *Comparative Aspects.* The most striking feature of the comparative aspect of these results is the nearly identical behavior of the reactive histidine in the enzymes from two widely divergent organisms. The histidines function identically or very similarly in terms of (a) apparent binding constants for diethyl pyrocarbonate, (b) first-order rate constants at pH 8.5 for inactivation and reactivation and for acylation and deacylation, (c) pH dependence of first-order rate constants, and (d) effects of active-center ligands on inactivation rates. These strongly suggest that the wheat and *E. coli* histidines are in very similar, if not identical, environments and perform identical functions. It is therefore surprising that the neighboring residues in the primary sequences of the two enzymes are totally lacking in homology. This could be explained by postulating divergent evolution of wheat-germ ATCase and the catalytic subunit of *E. coli* ATCase from a primordial ATCase ancestor, during which the essential histidine and its environment have been conserved for functional reasons. Under this hypothesis, the primary sequences immediately surrounding the histidines are not part of the conserved microenvironment. Presumably this environment is created in three dimensions by residues elsewhere in the chain. An alternative hypothesis is that of convergent evolution; this would require that ATCase activity evolve independently in plants and bacteria. Structural work on the wheat enzyme is not far enough advanced to test these hypotheses. Nevertheless, extensive primary homology between the *E. coli* catalytic chain and the ATCase domain of the Syrian hamster multifunctional CAD protein (Shigesada et al., 1985) provide a precedent for favoring divergent evolution between distantly related ATCases.

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Chemical Modification of Tryptophan Residues in *Escherichia coli* Succinyl-CoA Synthetase. Effect on Structure and Enzyme Activity†

Jesse Ybarra, A. R. S. Prasad, and Jonathan S. Nishimura*
Department of Biochemistry, The University of Texas Health Science Center, San Antonio, Texas 78284
Received April 16, 1986; Revised Manuscript Received July 22, 1986

ABSTRACT: Succinyl-CoA synthetase of *Escherichia coli* is an $\alpha_2\beta_2$ protein containing active sites at the interfaces between α- and β-subunits. The α-subunit contains a histidine residue that is phosphorylated during the reaction. The β-subunit binds coenzyme A and probably succinate [see Nishimura, J. S. (1986) *Adv. Enzymol. Relat. Areas Mol. Biol. 58*, 141–172]. Chemical modification studies have been conducted in order to more clearly define functions of each subunit. Tryptophan residues of the enzyme were modified by treatment with *N*-bromosuccinimide at pH 7. There was a linear relationship between loss of enzyme activity and tryptophan modified. At one tryptophan residue modified per β-subunit, 100% of the enzyme activity was lost. In this enzyme sample, one methionine residue in each α- and β-subunit was oxidized to methionine sulfoxide, although loss of enzyme activity could not be related in a linear manner to the formation of this residue. Subunits were prepared from enzyme that was inactivated 50% by *N*-bromosuccinimide with 0.5 tryptophan modified per β-subunit but with insignificant modification of methionine residues in either subunit. Small decreases in the tyrosine and histidine content were observed in the α-subunit but not in the β-subunit. In this case, modified β-subunit when mixed with unmodified α-subunit gave a population of molecules that was 50% as active as the refolded, unmodified control but was only slightly changed with respect to phosphorylation capacity and unchanged with respect to rate of phosphorylation. Relatively slight changes were observed in the phosphorylation capacity of the α-subunit both alone or when refolded with β-subunit. The results suggest that modification of a tryptophan residue in the β-subunit causes loss of catalytic activity but does not interfere with its ability to refold with the α-subunit to establish a conformation of the latter that is similar to that in the native enzyme. Failure of substrates and the CoA analogue desulfo-CoA to protect the enzyme against inactivation by *N*-bromosuccinimide would appear to indicate that the modified tryptophan residue is not at the active site.

Succinyl-CoA synthetase catalyzes the reaction

$$\text{NTP} + \text{CoA} + \text{succinate} \xrightleftharpoons{M^{2+}} \text{NDP} + P_i + \text{succinyl-CoA} \quad (1)$$

where NTP[1] and NDP are purine ribonucleoside triphosphate and diphosphate, respectively, and M^{2+} is a divalent metal ion. Covalent steps in catalysis by the enzyme are described in eq 2a–c (Nishimura & Grinnell, 1972; Bridger, 1974).

$$\text{NTP} + \text{enzyme} \rightleftarrows \text{enzyme-P} + \text{NDP} \quad (2a)$$

$$\text{enzyme-P} + \text{succinate} \rightleftarrows \text{enzyme·succinyl-P} \quad (2b)$$

$$\text{enzyme·succinyl-P} + \text{CoA} \rightleftarrows \text{enzyme} + \text{succinyl-CoA} + P_i \quad (2c)$$

Succinyl-CoA synthetase from *Escherichia coli* contains an $a_2\beta_2$ subunit structure. In the phosphorylation step (see eq 2a), the α-subunit is phosphorylated (Bridger, 1971). The β-subunit apparently contains at least part of the CoA binding site (Collier & Nishimura, 1978) and, perhaps, the succinate

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[1] Abbreviations: NTP, purine ribonucleoside triphosphate; NDP, purine ribonucleoside diphosphate; P_i, inorganic phosphate; ATP, adenosine 5′-triphosphate; NBS, *N*-bromosuccinimide; SCS, succinyl-CoA synthetase; Tris, tris(hydroxymethyl)aminomethane; EDTA, ethylenediaminetetraacetic acid.

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