An Aspartate Transcarbamylase Lacking Catalytic Subunit Interactions

STUDY OF CONFORMATIONAL CHANGES BY ULTRAVIOLET ABSORBANCE AND CIRCULAR DICHROISM SPECTROSCOPY*

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A modified form of aspartate transcarbamylase is synthesized by *Escherichia coli* in the presence of 2-thiouracil which does not exhibit homotropic cooperative interactions between active sites yet retains heterotropic cooperative interactions due to nucleotide binding. The conformational changes induced in the modified enzyme by the binding of different ligands (substrates, substrate analogs, a transition state analog, and nucleotide effectors) were studied using ultraviolet absorbance and circular dichroism difference spectroscopy. Comparison of the results for the modified enzyme and its isolated subunits to those for the native enzyme and its isolated subunits showed that the conformational changes detected by these methods are qualitatively similar in the two enzymes. Comparison of the absorbance difference spectra due to the binding of a transition state analog to the intact native or modified enzymes to those for the native enzyme and its isolated catalytic subunits showed that the modified enzyme and its isolated subunits possess, respectively, four and two high affinity sites for the inhibitor instead of six and three observed in the case of the normal enzyme and its isolated catalytic subunits. These results are correlated with the lower specific enzymatic activities of the modified enzyme and its catalytic subunits compared to the normal corresponding enzymatic species.

Aspartate transcarbamylase (carbamoylphosphate: L-aspartate carbamoyltransferase EC 2.1.3.2.) from *Escherichia coli* is currently undergoing extensive investigation as a model system to study conformational changes in regulatory proteins. This enzyme, which is made up of two trimers of catalytic chains and three dimers of regulatory chains, and whose subunit association is dependent on zinc (II) ions, shows the different types of interactions which are found in allosteric enzymes: (a) homotropic cooperative interactions between catalytic sites for the binding of aspartate; (b) heterotropic negative and positive interactions between regulatory and catalytic sites, allowing for feedback inhibition by CTP and stimulation by ATP; (c) two kinds of binding sites for the effector CTP which differ in affinity for this nucleotide and probably reflect the existence of homotropic negative cooperativity between regulatory sites. These regulatory processes in ATCase have been extensively reviewed and discussed (1, 2).

In the presence of 2-thiouracil, a modified ATCase is synthesized (2-ThioU-ATCase) in which the homotropic cooperative interactions between the catalytic sites are selectively abolished (3, 4). This modified enzyme, which contains, in normal proportion, the two kinds of subunits, shows Michalis-Menten kinetics for aspartate, but is still inhibited by CTP and

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stimulated by ATP. Consequently, a comparative study of the properties of 2-ThioU-ATCase versus those of ATCase may provide important information about structural or conformational features which are involved in the mechanism of homotropic interactions in ATCase.

Among the different methods used to study these conformational changes in ATCase, ultraviolet absorption and circular dichroism difference spectroscopy give interesting and concordant results. Upon binding of carbamylphosphate and succinyl (aspartate analog), an ultraviolet absorbance difference spectrum is observed showing perturbations in the environment of some tyrosine and tryptophan residues (5-9). Similar results are obtained using N-(p-hydroxyphenylacetyl)-1-aspartate (PALA), a "transition state analog" for the enzyme (6, 9). In this case, the very high affinity of the ligand allows a spectrophotometric titration of the number of catalytic sites. The various types of tyrosine and tryptophan perturbations provoked by the binding of carbamylphosphate, succinate, and the effectors CTP and ATP have been also investigated by circular dichroism difference spectroscopy (10-12). By comparing the effects of carbamylphosphate and succinate binding on native ATCase and isolated catalytic subunits, it was shown that some tyrosine residues, which are in an hydrophobic environment, are perturbed in the complete enzyme but not in the subunits (10). Although it was not established if these tyrosine residues belong to the regulatory subunit(s), it was suggested that this particular conformational change may be related to the existence of homotropic cooperative interactions between the catalytic sites. In the present report, the binding of substrates and analogs to the modified enzyme was investigated by ultraviolet and circular dichroism difference spectroscopy for comparison with the normal enzyme in order to determine if the lack of homotropic cooperative interactions could be related to an alteration of the conformational changes induced by the subunits.

This report also describes studies of the binding of the transition state analog PALA, to the modified 2-ThioU-ATCase versus ATCase and shows that a reduction in the number of high affinity PALA sites in the modified enzyme can be correlated with a reduction in specific enzymatic activity. In the course of this study, some information about the modification provoked by 2-thiouracil is presented.

MATERIALS AND METHODS

Chemicals and Enzymes – All chemicals were of the highest purity commercially available (3). PALA was generously provided by Drs. Kim Collins and George Stark, Stanford University. The partially diploid mutant of Escherichia coli used in these experiments for ATCase and 2-ThioU-ATCase production (3) was generously provided by Drs. J. Gerhart, University of California, Berkeley. ATCase and 2-ThioU-ATCase were purified as already reported (3) and both enzymes were dissociated with p-hydroxymercuribenzoate according to the procedure described by Hedrick and Smith (17). The running buffer contained 0.1% sodium dodecyl sulfate according to Laemmli (18). The running buffer contained in this case 0.1% sodium dodecyl sulfate. Gels were stained in 0.05% Coomassie brilliant blue in methanol/acetate acid/water (5:1:5) and destained in 20% methanol, 7.5% acetic acid charged with Dowex AG-1X8 (200 to 400 mesh) resin.

Determination of Absorption Coefficients – Absorbance spectra were obtained using a Cary 13 spectrophotometer at 20°. Solutions of the normal and modified enzymes in catalytic subunits between 0.5 and 3 mg/ml were dialyzed for 24 h at 4° against 0.04 M potassium phosphate, pH 7.0, containing 2 mM β-mercaptoethanol and 0.2 mM sodium/EDTA. Solutions of regulatory subunits at 4 mg/ml were dialyzed against the same buffer containing 0.2 mM zinc acetate in place of sodium/EDTA. The dialyzed solutions were filtered through Millipore filters (0.45 μ) and introduced into a 1-cm path length cuvette (Hellma Cells, Jamaica, N. Y.), while the reference cuvette contained filtered buffer.

In order to determine the protein extinction coefficients 200- to 500-nm light absorbance of samples containing 0.25 to 0.5 mg of protein were withdrawn from the sample cuvette just after recording the absorbance spectrum and hydrolyzed for 24 or 72 h at 110° by 2 ml of twice distilled 5.7 N HCl in the presence of 125 nmol of norleucine as an internal reference standard. Three amino acid analyses of each sample were performed either on a Beckman 12C analyzer (19) in the laboratory of Doctor Florence Lederer, Institut de Genétique Moléculaire, Centre National de la Recherche Scientifique or on a Technicon 3A-1 manual autoanalyzer in the Service de Biochimie, C.E.N.-Saclay.

PALA Titrations – Absorbance difference spectra due to PALA binding were obtained on a Cary 10 at 20°. Protein samples were extensively dialyzed against a 0.04 M potassium phosphate buffer, pH 7.0, containing 2 mM β-mercaptoethanol and 0.2 mM sodium/EDTA, and then were filtered through a Millipore filter (0.45 μ). The concentration of every dialyzed enzyme solution was determined by absorption difference spectra.
ThioU Catalytic Subunits—It has previously been noticed that 2-ThioU-ATCase possesses a specific activity which is significantly lower than that of normal ATCase (3). The specific activities of normal and modified ATCases and catalytic subunits were determined and are given in Table I where it is seen that the difference between the specific activities of catalytic and 2-ThioU catalytic subunits accounts for the 50% decrease in specific activity of 2-ThioU-ATCase compared to ATCase.

This lower specific activity is due to a difference in the catalytic chains in both species, and since, as shown in Table II, the sedimentation velocity experiments indicate that there is no significant difference in the sedimentation coefficients either between normal and modified enzymes, or between their catalytic subunits.

It has been observed that the specific activity of ATCase varies by 20% from one pure preparation to another (13, 20). The same observation was made in the case of 2-ThioU-ATCase.

Electrophoretic Mobility of 2-ThioU-ATCase and 2-ThioU Catalytic Subunits—It has been previously reported (3) that when 2-ThioU-ATCase is analyzed by electrophoresis on polyacrylamide gels in comparison with ATCase, it shows a slightly different migration. The electrophoretic mobilities of 2-ThioU catalytic subunits and catalytic subunits were measured under the same conditions. As seen in Fig. 1, 2-ThioU-ATCase and 2-ThioU catalytic subunits migrate slightly more slowly than the corresponding normal proteins. Since it was verified that there is no detectable difference between the electrophoretic mobilities of normal and modified regulatory subunits under the same conditions, it appears that a modification of the catalytic subunit is responsible for the difference in electrophoretic mobility between ATCase and 2-ThioU-ATCase.

These results indicate that there is a small difference of 20% in electrophoretic charge between the normal and the modified enzymes. This interpretation was verified by studying the variation of electrophoretic charge of the two kinds of proteins as a function of the acrylamide concentration according to Hedrick and Smith (17). Fig. 2 shows the result obtained when plotting the logarithm of the protein mobility relative to the dye front (Rm) versus acrylamide concentration. The straight lines corresponding to the two enzymes are parallel indicating a difference in charge but not molecular weight. A difference in molecular weight would have given two intersecting lines (17). This result is consistent with the absence of a detectable difference in $s_{\text{mean}}$ either between normal and modified ATCases or between their catalytic subunits (Table III).

ATCase and 2-ThioU-ATCase were also analyzed by electrophoresis on slab gels containing 0.1% sodium dodecyl sulfate, conditions under which both enzymes are dissociated into the constituent polypeptide chains which migrate according to their molecular weight. The gels stained were scanned in order to determine the relative intensities of the colored bands and thereby the relative amounts of catalytic and regulatory polypeptide chains. The results obtained (Fig. 3) show that no difference can be detected between normal and 2-ThioU-modified ATCases in terms of the relative proportion and molecular weight values of catalytic and regulatory chains.

**Ultraviolet Absorbance Spectra of Normal and Modified ATCases and Subunits**—The absorption spectra of 2-ThioU-

**TABLE I**

<table>
<thead>
<tr>
<th>Specific enzymatic activities of normal and 2-ThioU-modified ATCases and catalytic subunits</th>
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<tr>
<td>2-ThioU-ATCase (0.2 µg) and 2-ThioU catalytic subunits (0.1 µg), ATCase (0.1 µg), and catalytic subunits (0.1 µg) were assayed under the standard conditions. The specific activities are expressed in units per mg of proteins as defined under &quot;Materials and Methods.&quot; The specific activities per nmol of catalytic chains were calculated assuming that, like ATCase, 2-ThioU-ATCase contains six catalytic chains, of molecular weight 33,000, which represent 66% of the molecular weight of the total enzyme, and that 2-ThioU catalytic subunits are trimers of catalytic chains. Averages and standard deviations were calculated from five to seven determinations on a given preparation of enzyme.</td>
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<td>Specific activities</td>
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<tr>
<td>units/mg</td>
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<tr>
<td>ATCase</td>
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<tr>
<td>2-ThioU-ATCase</td>
</tr>
<tr>
<td>Catalytic subunits</td>
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<td>2-ThioU catalytic subunits</td>
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**TABLE II**

<table>
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<tr>
<th>Sedimentation coefficients of normal and 2-ThioU-modified ATCases and catalytic subunits</th>
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<tr>
<td>Solutions of ATCase (4 mg/ml), catalytic subunits (4 mg/ml), 2-ThioU-ATCase (2.9 mg/ml), and 2-ThioU catalytic subunits (2.5 mg/ml) in 0.04 M potassium phosphate buffer, pH 7.0, containing 0.2 mM EDTA and 2 mM β-mercaptoethanol, were centrifuged at 60,000 rpm at 20°C.</td>
</tr>
<tr>
<td>ATCase</td>
</tr>
<tr>
<td>2-ThioU-ATCase</td>
</tr>
<tr>
<td>Catalytic subunits</td>
</tr>
<tr>
<td>2-ThioU catalytic subunits</td>
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</tbody>
</table>

**Fig. 1.** Analysis of normal and modified ATCases and subunits by electrophoresis on polyacrylamide gels. 2-ThioU catalytic subunits (11 µg), catalytic subunits (12 µg), 2-ThioU-ATCase (10 µg), and ATCase (10 µg) were analyzed by electrophoresis on 7.5% polyacrylamide gels in Tris-glycine buffer (pH 8.3) as indicated under "Materials and Methods." 1, ATCase; 2, 2-ThioU-ATCase; 3, mixture of ATCase and 2-ThioU-ATCase; 4, catalytic subunits; 5, 2-ThioU catalytic subunits; 6, mixture of catalytic subunits and 2-ThioU catalytic subunits.
modified ATCase and subunits were recorded in comparison with the corresponding normal enzyme species. Fig. 4c shows that both enzymes present a maximal absorption at 279 nm, but that the extinction coefficient of 2-ThioU-ATCase is higher. In order to determine if this difference can be specifically attributed to one type of subunit, the absorption spectra of 2-ThioU catalytic and 2-ThioU regulatory subunits were recorded for comparison with the corresponding normal subunits. It can be seen in Fig. 4b that the absorption spectra of normal and 2-ThioU regulatory subunits are identical except for a slight difference for wavelength values below 270 nm, which is reminiscent of what is observed when apo-regulatory and zinc regulatory subunits are compared (21). It should be mentioned here that it has been already reported that 2-ThioU catalytic subunits are identical except for a slight difference for wavelength values below 270 nm, which is reminiscent of what is observed when apo-regulatory and zinc regulatory subunits are compared (21). It should be mentioned here that it has been already reported that 2-ThioU catalytic subunits are identical except for a slight difference for wavelength values below 270 nm, which is reminiscent of what is observed when apo-regulatory and zinc regulatory subunits are compared (21).

Interaction of N-(Phosphonacetyl)-L-Aspartate with 2-ThioU-ATCase

In contrast with ATCase, 2-ThioU-ATCase is not stimulated by PALA at low concentrations of aspartate. Under certain conditions, some substrate analogs can induce the homotropic cooperative interactions between catalytic sites in ATCase. At low concentrations, maleate, succinate, and PALA, which are competitive inhibitors of ATCase, stimulate its activity in the presence of low concentrations of aspartate (6, 22, 23). This is due to the fact that when they bind to the catalytic site, they are able, like aspartate, to induce the conformational change toward the structure which has the maximal affinity for the substrates. Since it has been previously established that 2-ThioU-ATCase does not show homotropic cooperative interactions between catalytic sites in the presence of aspartate (3, 4), such a stimulation by substrate analogs like PALA would not be predicted for this enzyme. The influence of PALA on the enzymatic activity of ATCase, catalytic subunits and 2-ThioU-ATCase is shown in Fig. 5. It is seen that 2-ThioU-ATCase behaves exactly like free catalytic subunits in that no stimulation of its activity is observed in sharp contrast to what happens in the case of normal ATCase whose activity is significantly stimulated by 1 mM PALA as already reported by Collins and Stark (6). This result provides additional evidence that homotropic cooperative interactions between catalytic sites do not occur in 2-ThioU-ATCase. In all cases, for concentrations of PALA higher than 3 mM, an inhibitory effect is observed. Collins and Stark (6) have shown that PALA inhibits the isolated catalytic subunits by competing with carbamylphosphate but not with aspartate. This competition process has been investigated here in the case of native ATCase and 2-ThioU-ATCase. The results, presented in Fig. 6 according to Lineweaver and Burk (24), show that PALA acts similarly on 2-ThioU-ATCase and catalytic subunits as a competitive inhibitor. It appears again in Fig. 6c that PALA stimulates the activity of normal ATCase, but at the same time acts as a competitive inhibitor towards carbamylphosphate. The initial velocity which extrapolates to 5 mmol/min/g in absence of PALA increases to 17 mmol/min/g in its presence. Since ATCase contains two-thirds of its mass as catalytic subunits, this maximal initial velocity corresponds to 25 mmol/min/g of catalytic subunits. It is interesting to note that this is very close to the value obtained in the case
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3

or

250 270 290 510 330

A. PML

FIG. 4. Ultraviolet absorbance spectra of 2-ThioU-modified and normal ATCases and subunits. The spectrophotometric measurements were made as described under "Materials and Methods." The extinction coefficients are expressed for molar solutions of catalytic chains, regulatory chains, and sum of both chains assuming that the molecular weights of these different species are 33,000, 17,000 and 50,000, respectively. a, ATCase, 2'-ThioU-ATCase, and their calculated difference spectrum; b, normal and 2'-ThioU-modified regulatory subunits spectra; c, normal and 2'-ThioU-modified catalytic subunits and their calculated difference spectrum.

TABLE III

Extinction coefficients of 2-ThioU-modified and normal ATCases and subunits

The extinction coefficients were calculated at maxima absorbance wavelengths (279 nm for native enzymes and catalytic subunits and 276 nm for regulatory subunits). Averages and standard deviations were calculated from seven or eight determinations on two different enzyme preparations in the case of native enzymes and catalytic subunits and from four or six determinations on two different preparations in the case of the regulatory subunits.

<table>
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<th>Extinction coefficient (mg/ml) ( \times 10^3 ) cm(^{-1} )</th>
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<tbody>
<tr>
<td>ATCase</td>
<td>0.59 ± 0.01</td>
</tr>
<tr>
<td>2'-ThioU-ATCase</td>
<td>0.73 ± 0.01</td>
</tr>
<tr>
<td>Catalytic subunits</td>
<td>0.73 ± 0.01</td>
</tr>
<tr>
<td>2'-ThioU catalytic subunits</td>
<td>0.85 ± 0.02</td>
</tr>
<tr>
<td>Regulatory subunits</td>
<td>0.37 ± 0.02</td>
</tr>
<tr>
<td>2'-ThioU regulatory subunits</td>
<td>0.38 ± 0.02</td>
</tr>
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</table>

of free catalytic subunits (Fig. 6b). The straight line corresponding to 0.9 \( \mu \)m PALA, a concentration which is high enough to ensure maximal stimulation of the enzyme, intersects the ordinate at the same maximal initial velocity as the straight line corresponding to a PALA concentration of 5.4 \( \mu \)m, a result which is characteristic of a competitive inhibition.

Titration of Catalytic Sites of 2'-ThioU-ATCase and 2'-ThioU catalytic subunits with PALA - As reported above, the specific enzymatic activities of 2'-ThioU-ATCase and 2'-ThioU catalytic subunits are about 50% of those of the corresponding normal enzymatic species. However, according to the structural studies, the catalytic polypeptide chain content of 2'-ThioU-ATCase and 2'-ThioU catalytic subunits does not differ from the catalytic polypeptide chain content of the corresponding normal proteins, that is to say three catalytic chains per catalytic subunit, and two catalytic trimers per molecule of native enzyme. Thus, the lower specific activity of 2'-ThioU-ATCase could result either from the presence of a normal number of less effective catalytic sites, or from a decreased number of normally active sites.

FIG. 5. Enzymatic activities of ATCase, 2'-ThioU-ATCase and catalytic subunits as a function of PALA concentration. ATCase (0.70 \( \mu \)g) (O—O), 2'-ThioU-ATCase (1.80 \( \mu \)g) (■—■), and catalytic subunits (0.50 \( \mu \)g) (Δ—Δ) have been incubated under the conditions described under "Materials and Methods" in the presence of 1 \( \mu \)m aspartate, 5 \( \mu \)m diithiolphosphate and increasing amounts of PALA.

In order to distinguish between these two possibilities, the spectrophotometric titration of the number of catalytic sites of 2'-ThioU-ATCase and its catalytic subunits was performed using PALA. This "transition state analog" synthesized and described by Collins and Stark (6) combines most of the structural features of the two substrates. Its binding to the catalytic sites gives rise to the same difference ultraviolet spectrum as that due to the binding of carbamylphosphate plus succinate. The very high affinity of PALA for the catalytic sites allows an accurate spectrophotometric titration of the number of sites (6, 9).

The magnitude of the difference spectrum is directly proportional to the amount of PALA bound allowing the determination of the number of binding sites at saturation, provided that the protein concentration is much greater than the dissociation constant of PALA.
In order to determine if 2-ThioU-ATCase contains a normal number of less active catalytic sites or a decreased number of fully active sites, this method was used for their titration, in comparison with normal ATCase. The results, presented in Fig. 7, show that 2-ThioU catalytic subunits and 2-ThioU-ATCase (Fig. 7, a and b), possess, respectively, two and four sites available for tight binding of PALA instead of three and six sites in the case of normal catalytic subunits and ATCase (Fig. 7, c and d).

It should be noted that this is a statistical result which might correspond to the existence of an heterogeneous population of catalytic trimers containing one, two, three, or no inactive monomers with an average of one per trimer. Since the specific activities of 2-ThioU-ATCase and 2-ThioU catalytic subunits are approximately 50% of the specific activities of the corresponding normal enzymatic species (Table I), the reduced number of high affinity PALA sites seen here coincides with the reduced specific activities.

Ultraviolet Absorbance Difference Spectra of 2-ThioU-ATCase and 2-ThioU Catalytic Subunits Due to PALA Binding – The ultraviolet absorbance difference spectrum observed for the binding of PALA to the catalytic subunits is identical to the one observed upon the binding of carbamylphosphate and aspartate (5, 6). These difference spectra were interpreted by Collins and Stark (5, 6) as a perturbation of some tryptophan and tyrosine residues. The ultraviolet absorbance difference spectrum produced by the binding of PALA to the 2-ThioU catalytic subunits was obtained and is presented in Fig. 8. The difference spectrum exhibits negative maxima at 279 and 287 nm which are characteristic of a blue-shifted tyrosyl absorbance spectrum. This result suggests that PALA binding induces in native ATCase but not in catalytic subunits a conformational modification in which some tyrosyl residues become more exposed to aqueous solvent or less buried in an hydrophobic environment. These tyrosyl residues may belong to the catalytic subunits or regulatory subunits, or both, and might be related to the existence of homotropic cooperative interactions.

Since 2-ThioU-ATCase does not show homotropic cooperative interactions between catalytic sites, it was interesting to determine if the conditional tyrosine perturbation could be detected in this modified enzyme compared to its catalytic subunits. Fig. 8 shows that the calculated difference between the two difference spectra for PALA binding to catalytic subunits or to ATCase. Although the observed difference spectra are complicated sums of red and blue shifts for both tryptophan and tyrosine chromophores, the overall difference spectrum appears to be slightly red-shifted for ATCase with respect to the one observed in the case of the catalytic subunits. However, the calculated difference spectrum exhibits negative maxima at 279 and 287 nm which are characteristic of a blue-shifted tyrosyl absorbance spectrum. This result suggests that PALA binding induces in native ATCase but not in catalytic subunits a conformational modification in which some tyrosyl residues become more exposed to aqueous solvent or less buried in an hydrophobic environment. These tyrosyl residues may belong to the catalytic subunits or regulatory subunits, or both, and might be related to the existence of homotropic cooperative interactions.
or 2-ThioU-modified regulatory subunits at 1.5 mol of PALA/mol of regulatory chain, no absorbance difference spectral perturbation was observed between 250 and 350 nm.

**CD Difference Spectra Due to Ligand Binding**

CD difference spectra between 250 and 400 nm due to the binding of ligands to the modified enzyme and to the modified subunits were obtained for comparison to previously published results for native ATCase and its isolated catalytic subunits (10).

In order to facilitate quantitative comparisons of CD difference spectra, studies of the binding of carbamylphosphate and succinate to the native ATCase and the isolated native catalytic subunit were repeated using the Jouan Dichrograph II.

The CD difference spectra which were obtained were qualitatively and quantitatively indistinguishable from those previously reported (10). Next the CD difference spectra due to the binding of carbamylphosphate to 2-ThioU-ATCase and 2-ThioU catalytic subunits were obtained. The observed CD difference spectra were qualitatively similar to those observed for the normal enzyme species and were indicative of the existence of similar tyrosyl and tryptophanyl perturbations in the modified enzyme species due to ligand binding. However, the maximum value of $\Delta(\theta)$ at 278 nm was 35% lower in the case of the modified enzymes.

CD difference spectra due to the binding of succinate in the presence of carbamylphosphate to ATCase and to the isolated catalytic subunit were identical to those previously reported.

![Fig. 7. Spectrophotometric titration of 2-ThioU-modified ATCase and catalytic subunits with PALA. $\Delta A$, the absorbance difference due to PALA binding was measured as the difference either between the maximum at 290 nm and the minimum at 285.25 nm in the case of native enzymes or between the maximum at 288.9 nm and the minimum at 284.5 nm in the case of catalytic subunits (see Fig. 8 for a typical difference spectrum); 2 to 3 mg/ml solutions of protein were used. These results correspond to two or three determinations. a, 2-ThioU catalytic subunit; b, 2-ThioU-ATCase; c, catalytic subunit; d, ATCase.](http://www.jbc.org/)

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FIG. 8. Absorbance difference spectra of 2-ThioU-modified and normal ATCases and catalytic subunits produced by saturating PALA. The spectra were obtained under the conditions described under "Materials and Methods." The extinction coefficient differences are expressed per mol of catalytic chains or sum of regulatory and catalytic chains, assuming that the molecular weights of these different species are 33,000 and 50,000, respectively. a, the observed absorbance difference spectrum due to PALA binding to 2-ThioU catalytic subunits (---) or to 2-ThioU-ATCase (----); b, the calculated differences between the two absorbance difference spectra in a; c, the observed absorbance difference spectra due to PALA binding to normal catalytic subunits (---) or to ATCase (----); d, the calculated differences between the two absorbance difference spectra in c.

(10). The binding of succinate to 2-ThioU-ATCase and 2-ThioU catalytic subunits gave CD difference spectra which were qualitatively similar to those for the native species but which exhibited a reduction of 50% in the maximal value of \(\Delta [\theta]\) at 290 nm. Thus, it appears that the binding of succinate and carbamylphosphate to the modified species results in tyrosyl and tryptophanyl perturbations which are very similar to those which exist in the native species. The 30 to 50% reduction in the maximal values of \(\Delta [\theta]\) most probably reflects a reduction in the number of binding sites for carbamylphosphate or succinate similar to that seen for the reduced binding of PALA.

CD difference spectra due to the binding of 0.12 mM CTP to ATCase and 2-ThioU-ATCase were obtained. The observed CD difference spectrum for the native enzyme was identical to the previously reported one (10). The results for 2-ThioU-ATCase were qualitatively the same as for the native enzyme, but the maximal value of \(\Delta [\theta]\) near 275 nm was reduced by 30%. This reduction may be correlated with a diminished sensitivity of 2-ThioU-ATCase to inhibition by CTP (3, 4). The observation reported here that 2-ThioU-ATCase is not stimulated by the transition state analog, PALA, at low concentrations of aspartate provides additional new evidence for the absence of homotropic cooperative interactions between catalytic sites in this modified enzyme. Thus, homotropic cooperative interactions between catalytic sites and heterotropic interactions between catalytic and regulatory sites in ATCase must correspond at least in part to distinct molecular mechanisms. That is, a simple two-state model cannot explain the properties of ATCase.

Several types of previous observations lead to the same conclusion that the conformational changes corresponding to the two types of interaction are not related in the sense that one is just the reverse of the other one. Buckman (25) reported that a given spin label probe allows the detection of the conformational changes corresponding to only one type of interaction. The detection of the other type of interaction requires the use of a different probe. By studying the binding of BrCTP and succinate in the presence of carbamylphosphate to ATCase using the temperature jump technique, Eckfeldt et al. (26) and Hammes and Wu (27) showed that the relaxation times are significantly different for these two ligands; when both ligands are added together to the enzyme in the presence

DISCUSSION

When Escherichia coli is derepressed for the biosynthesis of the enzymes of the pyrimidine pathway in the presence of 2-thiouracil, it synthesizes a modified ATCase (2-ThioU-ATCase) which does not show homotropic cooperative interactions between the catalytic sites, but which is still sensitive to the feedback inhibitor CTP (3, 4). The observation reported here that 2-ThioU-ATCase is not stimulated by the transition state analog, PALA, at low concentrations of aspartate provides additional new evidence for the absence of homotropic cooperative interactions between catalytic sites in this modified enzyme. Thus, homotropic cooperative interactions between catalytic sites and heterotropic interactions between catalytic and regulatory sites in ATCase must correspond at least in part to distinct molecular mechanisms. That is, a simple two-state model cannot explain the properties of ATCase.

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of saturating carbamylphosphate the two relaxation processes are observed (27). As quoted by the authors, such would not be the case if BrCTP and succinate would be acting in opposite directions on the same equilibrium; in this case, only the faster relaxation process would have been observed. Griffin et al. (11) have shown that upon binding of carbamylphosphate and succinate to the catalytic sites of native ATCase, a spectral perturbation which was assigned to the metal ion binding site is observed by circular dichroism spectroscopy (11); however, no perturbation of the metal ion binding site is observed upon CTP binding.

Upon binding of substrates or substrate analogs such as carbamylphosphate, succinate, and PALA to 2-ThioU-ATCase and 2-ThioU catalytic subunits, the spectrophotometric perturbations observed by ultraviolet absorbance and circular dichroism difference spectroscopy do not qualitatively differ from what is observed in the case of normal ATCase and catalytic subunits. This suggests that those conformational changes due to substrate binding which result in detectable tryosyl and tryptophanyl perturbations are qualitatively the same in the modified and normal enzymes. Therefore, the lack of homotropic interactions in 2-ThioU-ATCase is not the result of a detectable alteration of the conformational changes involving the perturbed tryosyl and tryptophanyl residues.

Previous studies of the ultraviolet absorbance difference spectrum due to the association of normal isolated catalytic and regulatory subunits indicated that tryosyl and phenylalanyl residues become buried in a hydrophobic environment (10). It is tempting to speculate that the exposure to solution of similar residues induced by PALA binding to the native enzyme but not the isolated catalytic subunits is the reversal or loss of subunit contacts affecting these same aromatic amino acid residues. This interpretation is consistent with the concept that subunit association introduces constraints which are decreased in the course of positive homotropic interactions. Positive cooperativity would thus involve the loss of constraining interactions. Since both the modified and normal forms of ATCase exhibit these phenomena, such conformational changes are not sufficient by themselves to explain homotropic cooperativity.

Upon binding of CTP to 2-ThioU-ATCase, the circular dichroism difference spectrum obtained is again qualitatively the same as that obtained with normal ATCase except for a minor quantitative difference. Interestingly, this quantitative reduction in the circular dichroism difference spectra due to ligand binding is accompanied in the modified enzyme species by diminution of the enzymatic specific activity, the number of high affinity PALA binding sites and the efficiency of CTP inhibition.

Since the various conformational changes reflected in aromatic amino acid spectral perturbations upon the binding of the different ligands, i.e. carbamylphosphate, succinate, PALA, and CTP, to the two kinds of modified subunits appear to be normal, the lack of homotropic cooperative interactions in the modified enzyme might result from a defect in subunit interactions involving the intersubunit bonding domains. The fact that the thermosensitivity of 2-ThioU-ATCase is the same as that of free catalytic subunits, in contrast to the greater heat stability of the native ATCase, would support this hypothesis (3).

By studying the properties of the hybrids made up of subunits derived from normal and modified ATCases, it was previously shown that the lack of interactions can be attributed to a modification of the regulatory subunits (4). The data in this paper show that the catalytic subunits are also modified in 2-ThioU-ATCase, and that these modifications are responsible for a different electrophoretic mobility, for a higher extinction coefficient, for a decreased specific enzymatic activity, and for a reduction in the number of PALA binding sites. Since 2-ThioU regulatory subunits possess the same electrophoretic mobility and the same extinction coefficient as normal regulatory subunits, catalytic and regulatory chains may be modified in a different way in 2-ThioU-ATCase. These modification(s) could result either from a miscoding provoked by a slight incorporation of 2-thiouracil in messenger RNA or transfer RNA, or from a post-translational chemical modification by 2-thiouracil itself or a product of its transformation. Both phenomena may happen in the same time. Another possible effect of 2-thiouracil has been discussed recently by Ridge et al. (28) who suggested that this nucleotide might perturb the folding of ATCase during its biosynthesis.

The specific activity of 2-ThioU-ATCase is about 50% that of the normal enzyme, and it appears that the catalytic subunits are entirely responsible for this difference since the isolated 2-ThioU catalytic subunits exhibit the same reduced specific activity. It is interesting to note that the reassocation of 2-ThioU catalytic subunits with normal regulatory subunits does not alter this reduced activity (4). The reduced number of high affinity PALA sites in 2-ThioU-ATCase and 2-ThioU catalytic subunits suggests that the lower specific activity is most likely due to a defect of one out of three catalytic sites. Since this number of sites is an average, 2-ThioU-ATCase may be an heterogeneous population of molecules containing varying proportions of defective catalytic chains per catalytic trimmer. Further studies to determine the chemical nature of the modification provoked by 2-thiouracil are currently under investigation.

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