The Influence of Quaternary Structure on the Active Site of an Oligomeric Enzyme

CATALYTIC SUBUNIT OF ASPARTATE TRANSCARBAMOYLASE*

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The catalytic subunit of aspartate transcarbamoylase from Escherichia coli reacts readily with 2,4,6-trinitrobenzenesulfonate, resulting in the loss of enzymatic activity. Substrates and substrate analogs protect the enzyme in a competitive manner, indicating that the loss of activity is due to modification of active-site residues. This conclusion was confirmed by fractionation of trypic digests of the modified protein followed by the identification of active-site lysines 83 and 84 as the modified residues.

When three trinitrophenyl groups are incorporated per catalytic trimer, 70% of the activity is lost. The modified protein retains the sedimentation velocity and electrophoretic properties of the native catalytic subunit and can associate with regulatory subunit to form a holoenzyme-like molecule. The trinitrophenylated catalytic trimers have two strong absorption bands at 345 and 420 nm which serve as sensitive spectral probes in difference-spectroscopy experiments. Results from such experiments show that 1) the modified trimeric enzyme binds active-site ligands; 2) dissociation of the trimer into compact, highly structured monomers gives a spectral response distinguishable from that observed when the chains are completely unfolded; and 3) even though dissociation of the trimers to folded monomers causes the complete loss of enzyme activity, the resulting monomers still retain the ability to bind the bisubstrate analog N-(phosphonacetyl)-L-aspartate. These results indicate that the active site must be at least partially formed in the absence of any quaternary structure.

Interactions between subunits are required for activity in many oligomeric proteins. Although a large number of proteins consist of multiple subunits, the relationship between activity and quaternary structure often is not well understood, especially for oligomers consisting of a small number of identical subunits. In this paper, we attempt to determine how subunit interactions contribute to the enzymatic properties of the trimeric catalytic subunit of Escherichia coli aspartate transcarbamoylase (EC 2.1.3.2, carbamoylphosphate:1-aspartate carbamoyltransferase).

Considerable research on the allosteric properties of ATCase has focused on subunit structure and the ligand-promoted conformational changes responsible for its regulatory properties. The activity of the holoenzyme is cooperative with respect to the substrates carbamoylphosphate and aspartate, whereas the purified C subunit is a Michaelian enzyme which does not exhibit the characteristic inhibition by CTP and activation by ATP observed with intact ATCase. However, the correlation between the enzymatic properties of C subunit and its quaternary structure is not well understood. Several experiments suggest that the trimeric form of C subunit is required for activity.

The reconstitution of active C trimers from unfolded polypeptide chains (produced by treatment of the protein with urea or guanidinium chloride) involves an initial folding process followed by a series of association reactions. The rate at which activity is regained correlates strongly with the rate of trimer formation. Incubation of C subunit with chaotropic salts such as NaSCN or NaCIO, results in formation of inactive, folded monomers. Restoration of activity after dilution of the salt is coincident with the assembly of trimers from monomers.

As yet no evidence has been reported indicating that monomers exhibit any functional aspects of the trimeric subunit. It is not clear what structural features are responsible for the lack of function. How different are the active-site environments in the trimeric and monomeric forms? Does the active site exist in a monomer? How are ligand binding properties affected by dissociation of C trimers?

Experiments utilizing chemical modification of C subunit have identified several residues which are important for activity. Reactions which cause inactivation of the enzyme include pyridoxylation of lysine 84 (7, 8), alklylation of lysines 83, 84, and 232 by bromosuccinate (9), and nitration of tyrosine 165 (10). Many, but not all, modifications of cysteine 46 result in loss of activity (11-13). Other residues important for catalytic activity have been implicated, including 1 arginine (14) and 2 histidines (7), but have not been specifically identified. It is important to note, however, that in much of this work there has been no convincing demonstration that inactivation resulting from chemical modification is the direct consequence of altering an active-site residue. For many chemical derivations...
tives, the loss of enzyme activity might instead be the result of an indirect effect stemming from a conformational change.

X-ray diffraction studies of unliganded ATCase at 3.0-A resolution (15, 16) indicate that lysine residues 83 and 84 are located at the interfaces between pairs of catalytic chains. Distance measurements between tyrosine 165, cysteine 46, and lysines 83 and 84 within one chain and between pairs of chains led to the suggestion that the active site involves the joint participation of residues from adjacent polypeptide chains (16). However, the distances between the side chains of these residues range from 8 to 30 A, and, as such, substantial conformational changes would be required to bring the side chains close together. Thus far, the high-resolution crystallographic structure has been determined for only the low-affinity, T-state form of the enzyme. Until the structure of the complex between ATCase and the tight-binding bisubstrate analog PALA is determined so that the active site can be located unambiguously, questions remain concerning the extent to which the crystal structure of unliganded ATCase can be correlated with the chemical modification results for isolated C subunit.

In the work reported here, C subunit was modified by reaction with 2,4,6-trinitrobenzenesulfonate to produce a largely inactive derivative containing a sensitive spectral probe. This modified protein was used to monitor changes in the active-site environment upon the binding of ligands or upon perturbation of the tertiary and quaternary structure of the enzyme.

**MATERIALS AND METHODS**

Detailed experimental procedures for measuring the reaction between C subunit and TNBS and characterizing the modified derivative CTNP are described in the Miniprint Supplement. This supplement also presents results on the isolation and identification of the TNF-peptides produced by treatment of CTNP with trypsin.

**RESULTS**

TNBS Inactivates C Subunit and Active-site Ligands Inhibit the Reaction—In preliminary experiments on the chemical modification of C subunit with TNBS, Bothwell (17) showed that the enzyme was 99% inactivated after reaction with a large excess of the reagent. Moreover, the kinetic analysis of the biphasic reaction suggested that 1 lysine residue was much more reactive than the other 14 lysines and that the more rapid reaction was virtually eliminated if active-site ligands were present. In extending these studies, we have found that C subunit lost 77% activity in 5 min and 97% activity after 30 min of reaction with TNBS in molar excess of approximately 200-fold. In contrast, only 11 and 67% inactivation occurred at corresponding times if the reaction mixture contained 1 mM carbamoylphosphate and 5 mM succinate. Although these active-site ligands protected the enzyme, the inactivation due to the TNBS reaction could be attributed to either a direct effect of modification of active-site residues or an indirect effect stemming from conformational changes due to reaction at regions distant from the active site. In order to resolve this ambiguity, we conducted more detailed kinetic studies on inactivation rates at several TNBS concentrations. As seen in Fig. 1A, the inactivation of C subunit with excess TNBS (20–200-fold over active sites) follows pseudo first-order kinetics, and the rate is dependent on TNBS concentration. If the reactions are performed in the presence of low amounts of carbamoylphosphate (10 μM) and succinate (500 μM), the rates of inactivation are reduced significantly (Fig. 1B). When the rate constants from these experiments are plotted against TNBS concentration (Fig. 2A), the curves approach plateau levels. A plot of $k^{-1}$ versus [TNBS] is linear both with and without substrates, and the extrapolated lines intersect at the ordinate (Fig. 2B). Thus, carbamoylphosphate and succinate are competing directly to prevent inactivation, as would be expected if the modification involves residues at the active site (18).

Although each c chain contains 15 lysine residues, many are unreactive toward TNBS under the conditions used in these experiments. Treatment of the unliganded protein with a 200-fold excess of TNBS/active site for 24 h resulted in the modification of only 4.8 lysine residues/chain. In the presence of 10 eq of the bisubstrate analog PALA, only 3.0 residues were modified. Thus, 2 reactive lysine residues are protected by PALA.

**TNBS Modifies Lysine Residues 83 and 84 to Cause Inactivation**—Because the kinetic studies of the inactivation of C

![Fig. 1. Inactivation kinetics of C subunit by TNBS. C subunit (final concentration 0.5 mg/ml) in 20 mM triethanolamine acetate buffer, pH 8, was incubated at 25 °C with or without active-site ligands (10 μM carbamoylphosphate and 500 μM succinate). At zero time, a freshly prepared solution of TNBS in buffer was added to give the desired final concentration. At the times indicated, a 10-μl aliquot was removed and diluted in 990 μl of cold assay buffer to stop the modification. Residual activities were determined using 4 mM carbamoylphosphate and 25 mM aspartate and are expressed as a percentage of a control reaction utilizing no TNBS. A, no ligands; B, plus ligands. O, 0.33 mM TNBS; ●, 0.5 mM TNBS; ▲, 1.0 mM TNBS; Δ, 2.0 mM TNBS; ■, 3.33 mM TNBS.
To TNBS indicated the presence of one or several rapidly reacting lysine residues, we attempted to identify these residues by increasing the specificity of the chemical modification. This was accomplished by modifying C subunit with only a slight (10%) excess of the reagent. The derivative was then characterized in terms of residual activity and extent of modification, and identification of TNP-lysine residues.

The extent of inactivation of the enzyme is correlated to the number of modified residues, as shown in Fig. 3. The activity decreases linearly with the extent of modification, such that a derivative with an average of 1 TNP-lysine/c chain has about 30% the activity of unmodified C subunit. Even in the presence of PALA, which is known to enhance the rate of trimer assembly and strengthen interactions between chains, CTNP dissociates nearly all the protein to folded monomers. Finally, CTNP retained the ability to bind active-site ligands, as described in the next section.

**CTNP Contains a Sensitive Spectral Probe at the Active Site**

The spectral properties of CTNP are shown in Fig. 4. Absorption in the region between 250–300 nm is increased slightly above the spectrum of native C subunit. The major effect of the modification is seen in the region of 300–550 nm, where CTNP has two major absorption bands with maxima at 345 and 420 nm. These bands are perturbed when the modified

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**Fig. 2. Kinetic analysis of inactivation reaction.** Pseudo first-order kinetic constants k were derived from the slopes of the lines in Fig. 1. A, rate constant dependence on TNBS concentration; B, double-reciprocal plots of the data from A, data obtained in the absence of active-site ligands; △, data obtained in the presence of ligands.

**Fig. 3. Specificity of TNBS reaction.** C subunit (10–20 mg/ml) in 20 mM triethanolamine acetate, pH 8.5, containing 0.2 mM EDTA, was reacted at 0°C with a 10% excess of TNBS over active sites. At times between 0 and 4 h, aliquots were removed and characterized in terms of residual activity and extent of modification as detailed in the Miniprint Supplement. The line represents the linear least-squares fit of the data.

**Fig. 4. Absorption spectra of modified and native C trimers.** Spectra were obtained using a buffer of 25 mM imidazole acetate, pH 7.0, containing 2 mM 2-mercaptoethanol and 0.2 mM EDTA. The dashed line represents the spectrum of native protein, while the solid line is the spectrum of CTNP with 1.0 modifications/c chain.
Affect the Chromophore—Previous studies have shown that protein binds carbamoylphosphate, succinate, and PALA, as was then recorded. In equal volume of buffer was added to the reference cell. The difference concentration of 10 mM. Succinate by itself did not give any difference peaks near 290 nm are also observed with unmod- aldons were observed with other perturbing solvents (data not shown): 1.75 M NaClO₄ dissociated the trimer and gave a spectrum similar to that in NaSCN, while urea in

![Fig. 5. Effect of active-site ligands on the chromophore.](image)

The base-line was recorded with CTNP (1 mg/ml) in each cell. A small aliquot of concentrated ligand was added to the sample cell, and an equal volume of buffer was added to the reference cell. The difference was then recorded. In A, the solid line represents the addition of carbamoylphosphate to a final concentration of 4 mM; the dashed line is due to the addition of succinate (10 mM final concentration) in the presence of carbamoylphosphate. B shows the response due to the addition of PALA (100 μM).

FIG. 5. Effect of active-site ligands on the chromophore. The base-line was recorded with CTNP (1 mg/ml) in each cell. A small aliquot of concentrated ligand was added to the sample cell, and an equal volume of buffer was added to the reference cell. The difference concentration of 10 mM. Succinate by itself did not give any difference peaks near 290 nm are also observed with unmodified C subunit and are due to perturbation of aromatic side chains of the protein (21, 22). The addition of 10 mM succinate in the presence of carbamoylphosphate resulted in a small perturbation centered at about 340 nm (Fig. 5A) and a peak near 290 nm characteristic of native protein (21). Further additions of succinate resulted in larger peaks at 340 nm, suggesting that saturation had not been reached at a concentration of 10 mM. Succinate by itself did not give any difference spectrum.

Changes in the Tertiary or Quaternary Structure of CTNP Affect the Chromophore—Previous studies have shown that chaotropic salts such as NaSCN and NaClO₄ dissociate C subunit to highly folded, inactive monomers which have spectral and hydrodynamic properties similar to C trimers (6). In contrast, guanidinium chloride or urea produces unfolded polypeptide chains. The difference spectra in Fig. 6 show the large changes due to perturbation of CTNP by 1.25 M NaSCN or 4 M guanidinium chloride. The negative peak near 285 nm in each spectrum is similar in location and magnitude to that observed for unmodified protein and corresponds to the perturbation of aromatic side chains (6). However, in the region from 300 to 550 nm, the two perturbants yield spectra with major differences in peak amplitudes, locations, and signs. Analogous results were observed with other perturbing solvents (data not shown): 1.75 M NaClO₄ dissociated the trimer and gave a spectrum similar to that in NaSCN, while urea in sufficient concentration to denature the protein (4.5 M) yielded a result similar to that observed with guanidinium chloride. We interpret these results to mean that the environment of the chromophore in the folded monomeric species is distinct from that of both the trimeric and the unfolded protein.

The dependence of the spectral response of CTNP at 440 nm as a function of NaSCN concentration is seen in Fig. 7. There are two distinct phases in the titration curve. The first, from 0 to 1.25 M NaSCN, results in a positive peak at 440 nm. We interpret this to correspond to the transition from trimer to folded monomer, in accordance with the sedimentation velocity data presented above. The second phase occurs from 1.5 to 4 M NaSCN. At such high salt concentrations, unfolding of the chains is probably taking place. This is supported by the fact that the difference spectrum caused by 4 M NaSCN is nearly identical to that produced by the same concentration of guanidinium chloride.

Folded Monomers of CTNP Bind PALA—Because the binding of carbamoylphosphate and PALA to CTNP yields difference spectra of such large magnitude (Fig. 5), we were able to measure ligand binding to the monomeric and trimeric forms of the protein by spectral titration. The values for the disso-

![Fig. 6. Effects of NaSCN and guanidinium chloride on CTNP.](image)

FIG. 6. Effects of NaSCN and guanidinium chloride on CTNP. Difference spectra were measured for CTNP in 1.25 M NaSCN or 4 M guanidinium chloride versus CTNP trimers in standard Tris buffer at 10 °C. The base-line was recorded before mixing the sample tandem cell. The sample was then mixed, and after 20 min, the difference spectra were recorded. The solid line represents the spectrum in guanidinium chloride, while the dashed line is the spectrum in NaSCN.

![Fig. 7. Dependence of spectral response on NaSCN concentration.](image)

FIG. 7. Dependence of spectral response on NaSCN concentration. Difference spectra were determined as described in the legend to Fig. 5 using varying concentrations of chaotropic salt. The magnitude of the change at 440 nm was determined and is plotted versus NaSCN concentration.
cation constants are given in Table I. Intact C_{TNP} trimers in dilute buffer have an affinity for carbamoylphosphate (11 μM), which is reduced to about 25% that of the native subunit (3 μM). Thus, the bulky TNP group interferes only slightly with the binding of carbamoylphosphate. In contrast, PALA binding is reduced to about 0.3% (from 3 nM for native C subunits to 1 μM for C_{TNP} trimers). Thus, trinitrophenylation lowers the affinity of the trimer for PALA much more than it affects the binding of carbamoylphosphate.

Solutions of modified, folded monomers were prepared by incubation in 1.25 M NaSCN and were tested for the ability to bind active-site ligands. The addition of PALA (25 mM) to this solution resulted in the difference spectrum shown in Fig. 8. Carbamoylphosphate at a concentration of 25 mM did not perturb the chromophore. The affinity for PALA under these conditions was determined by spectral titration, giving a dissociation constant of approximately 25 mM (Table I). Al-fected by the presence of NaCl or NaSCN. Both of these salts perturb the chromophore. The affinity for PALA under these conditions was determined by spectral titration, giving a dissociation constant of approximately 25 mM (Table I). Although this seems to be an extremely weak affinity, Table I also gives a comparison of binding affinities for the modified protein in 1.25 M solutions of several salts, showing that carbamoylphosphate and PALA binding can be strongly af-fected by the presence of NaCl or NaSCN. Both of these salts are inhibitors of carbamoylphosphate in enzyme assays (24, 25), but only NaSCN causes dissociation of the trimer. Becaue the binding of PALA in NaSCN is only slightly weaker than in the same concentration of NaCl (25 versus 6 mM), the results indicate that the monomers still retain an affinity for PALA which is not very different from that of trimers at comparable ionic strength.

**TABLE I**

<table>
<thead>
<tr>
<th>Sample</th>
<th>Salt, mM</th>
<th>$K_i$ for salt, mM</th>
<th>$K_i$ for CbmP, mM</th>
<th>$K_i$ for PALA, mM</th>
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<td>C</td>
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<td></td>
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<td>4*</td>
<td>NC</td>
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</table>

* Determined using the method of Porter et al. (23).

**FIG. 8.** Effect of PALA on C_{TNP} monomers. Modified protein at 1 mg/ml in 1.25 M NaSCN was present in both cells. The baseline was recorded and then PALA was added (final concentration of 25 mM) to the sample cell, while an equal volume of buffer was added to the reference cuvette. After 10 min, the difference was recorded.

**DISCUSSION**

**Characteristics of C_{TNP}**—Studies of the kinetics of inactivation (Figs. 1 and 2) and the correlation of modification and inactivation (Fig. 3) support the hypothesis that TNBS reacts with active-site residues of C subunit. The modified residues were identified by peptide mapping as lysines 83 and 84. The specificity for these residues may be attributable to the preferential reaction of active-site residues with negatively charged reagents (26). For TNBS, the full negative charge of the sulfonate group plus the polarizability of the nitro groups may contribute to the specificity of the reaction. Alternatively, lysines 83 and 84 could share one low pK, resulting in an unassembled reactive amino group. We have not attempted to distinguish between these possibilities. Although TNBS generally reacts non-specifically with exposed lysine residues of proteins (27), ADP glucose synthetase has also been specifically modified by this reagent (28).

The sensitivity of the chromophore in C_{TNP} allows the detection of even small changes in the active-site environment by monitoring the absorption spectrum between 300 and 550 nm. Furthermore, C_{TNP} is very similar to the native protein, since the modification does not affect physical properties such as sedimentation and electrophoretic behavior. Preparations of C_{TNP} described here have one modification/c chain and retain about 30% activity. Since essentially all polypeptide chains had incorporated a TNP group, this enzymatic activity is apparently inherent in the derivative rather than due to unmodified protein in the preparation. The heterogeneity of the derivative might complicate certain experiments. One example is the observed perturbation at 340 nm by succinate which could be due to binding to one site, followed by communication of the binding to other sites within the trimer. Such an effect has been reported for partially pyridoxylated C subunit (8).

**Ligand Binding Properties of C_{TNP} Trimers**—The addition of active-site ligands carbamoylphosphate and PALA to C_{TNP} results in difference spectra of large magnitude (Fig. 5). The proximity of these ligands to the TNP group may account for the magnitude of these difference spectra. Table I gives the dissociation constants for carbamoylphosphate and PALA determined by spectral titration of the modified protein. There is a trend toward lower affinities for both carbamoylphosphate and PALA binding to C_{TNP} trimers in dilute buffer, but PALA is affected much more strongly. Whereas the binding of carbamoylphosphate to the modified protein was reduced to 25% for the native protein, the affinity for PALA was decreased to 0.3% (Table I). This indication that the asparyl portion of PALA is bound weakly is consistent with results which indicate a lower affinity of C_{TNP} for succinate in the presence of carbamoylphosphate. The simplest explanation of these results is that the TNP ring occupies at least part of the asparate-binding site. A derivative of C subunit which is pyridoxylated at lysine 84 also binds carbamoylphosphate wel1* but PALA poorly (7). On the basis of the affinity for ligands of these two chemically modified proteins, the conclusion can be drawn that lysine 84 plays an important role in the binding of aspartate, succinate, and PALA.

**Effects of Tertiary and Quaternary Structure on the Active Site**—The TNP chromophore is sensitive to both the tertiary and quaternary structure in trinitrophenylated C subunit (Fig. 6). The region of the spectrum between 300 and 550 nm was strongly perturbed when the modified protein was exposed to dissociating or denaturing solvents (Fig. 5). 1.25 M NaSCN (or 1.75 M NaClO₄) perturbed the chromophore in a manner

* Y. R. Yang, unpublished results.
quite different from the effects of 4 M guanidinium chloride (or 4.5 M urea). Previous studies (6) and control experiments here show that the first two reagents promote dissociation of the trimer to a folded monomeric state, while the second set of solvents both dissociate and unfold the protein. A thorough study of the effects of agents such as NaSCN on model compounds led to the prediction that hydrophobic interactions are weakened due to the solvation of residues which are normally buried (29). Burns and Schachman (20) found that the rate of polypeptide-chain exchange in C trimers is much slower at 25 °C than at 0 °C, implying that hydrophobic interactions constitute the major force holding the chains together. Titration of CTNp with NaSCN (Fig. 7) indicates that disruption of the forces between chains occurs at salt concentrations below 1.25 M, while at higher concentrations (4 M) the chains are denatured. It is somewhat unusual that we can separate these effects by controlling the type and concentration of the perturbed unit; similar responses are known for only a few other proteins (30-33).

**PALA Binding to Monomers of CTNp**—Tests using several anions showed that effects on ligand binding to CTNp are mainly due to competition rather than increases in ionic strength (Table I). Acetate ion, which is a very weak inhibitor of C subunit (24), lowers the affinity of CTNp for carbamoylphosphate to 33% at a salt concentration of 1.25 M. The effect on PALA was somewhat stronger, namely a reduction in binding strength to 10%. However, an equal concentration of NaCl led to much more dramatic results; the dissociation constant for carbamoylphosphate binding rose from 11 μM to 7 mM, while that for PALA increased from 1 μM to 6 mM. The competitive effect of NaCl on carbamoylphosphate binding can be predicted using the equation $K_{app} = K(1 + [I]/K_I)$, where $K$ equals the dissociation constant in the absence of inhibitor, [I] is the inhibitor concentration, and $K_I$ is the inhibition constant. From a value of $K = 35$ μM (the binding constant in 1.25 M NaOAc), $K_{app}$ is estimated to be 5 mM, in reasonable agreement with the measured value of 7 mM.

CTNp in the presence of 1.25 M NaSCN bound PALA (Fig. 8), and the binding constant under these conditions was estimated to be 25 mM (Table I). Much of the lowering in affinity is due to competition by SCN$^-$; NaSCN inhibits the binding of carbamoylphosphate to native C subunit with an inhibition constant of 4 mM. Since carbamoylphosphate and PALA in solutions of high salt concentration bind to CTNp with approximately equal affinities, SCN$^-$ presumably inhibits its PALA binding also. Based on these assumptions, $K_{app}$ for PALA binding to CTNp in 1.25 M NaSCN is about 4 mM, or only one-sixth the observed dissociation constant. Thus, the bulk of the effect on the ability of CTNp in 1.25 M NaSCN to bind PALA can be attributed to competition by thiocyanate ion rather than alterations in protein structure caused by this agent. Another estimate of the PALA affinities of trimers versus monomers based on titration results in 1.25 M NaCl or 1.25 M NaSCN indicated a 75% decrease due to dissociation of the trimer. The active site of C monomer must retain a significant portion of its ligand binding capability even in the absence of quaternary structure. PALA is also known to enhance the rate of trimer assembly of native C subunit from monomers produced by treatment with NaNCS (6). If more than one species of folded monomers exist, PALA binding might, for example, cause a conformational change in the protein to a structure which is competent for assembly. A conformational change of this type could give rise to the difference spectrum observed when PALA is added to CTNp in 1.25 M NaSCN (Fig. 8).

Why are monomers inactive? The experiments reported here do not provide a complete answer. Even though monomers produced by incubating CTNp in 1.25 M NaSCN still bind the bisubstrate ligand PALA, the addition of carbamoylphosphate did not cause a difference spectrum. The loss of enzymatic activity in the monomeric protein indicates that some of the residues essential for catalysis are displaced. Clearly, this could be attributed directly to the dissociation of the trimers if the residues constituting the active site are located on two different, adjacent polypeptide chains. Alternatively, the absence of activity in the monomers could be attributed to subtle changes in their tertiary structure as compared to their structures when incorporated into trimers. It is possible that stabilization of the active form of the protein requires indirect effects of interactions between chains. Since dissociation of modified trimers to monomers by NaSCN caused only a small decrease in the affinity for PALA (compared to the affinity of the modified trimers in NaCl), we conclude that a substantial portion of the active site is intact within a single folded polypeptide chain.

**REFERENCES**

Active Site of ATCase Catalytic Subunit

6852-6860

Additional references are found on p. 13913.
This supplement provides detailed procedures for the analysis of the reaction between Cys and TNBS, and methods for characterizing the modified protein Cgp. The isolation and identification of TNP-containing species is delineated. The isolation and identification of TNP-containing peptides produced by treatment of Cgp with trypsin are also described.

**Isolation and Identification of TNP-Containing Peptides**

**Chemicals**—TNBS was purchased from J. T. Baker and recrystallized from ethyl acetate/chloroform before use. Neohydrin was from RBI Laboratories, carbamylphosphate from Boehringer-Mannheim, and PAPA was kindly provided by Dr. Robert E. Engel, Developmental Therapeutics Program, National Cancer Institute. Guanidinium chloride (ultrapure) came from Worthington. r-TNF-α, Lysine hydrochloride, and TNP-trypsin were from Bio-Rad Laboratories, and Tris-phosphate, pH 9.5, containing 1 mM EDTA, was kindly provided by J. Roth.

**Preparation of TNP and Its Subunits**—TNP was prepared according to the method of Gerhart and Holowczuk (1) from ethyl acetate/TMS 363 (tms 75/21). This strain (obtained from J. Roth) was why (4). Subunits of ATCase were also described.

**Sequence Analysis of the First Four Residues of Peptide I** yielded an order of Gly-Arg-Ala-Arg, which corresponds to residues 46-49 of the catalytic chain (6, 9, 10). The predicted tryptic peptide with this N-terminal sequence would extend from Lys 46 to Lys 84. The results of quantitative amino acid analysis of peptide I are shown in Table 1A. Seventeen of 19 entries (all except Cys) correspond exactly to the calculated values for a peptide with N-terminal Lysine 83 side chain.

**Sequence Analysis of the First Four Residues of Peptide II** gave an N-terminal sequence of Tyr-Cys-Glu-Thr, which corresponds to residues 44-48 of the catalytic chain. Lysine 84 must be the modified residue in this peptide.

**Supplementary Material**

**Table 1A**

<table>
<thead>
<tr>
<th>Peptide</th>
<th>Cysamide acid analysis of TNP-peptides I and II</th>
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**References**


**Figure 1A**

**Elastin:** SEAC-cellulose chromatography of a tryptic digest of Cgp. Pooled fractions from Sephadex G-50 were applied to a 1.5 x 50 cm SEAC-cellulose column equilibrated in 100 mM ammonium bicarbonate, pH 8.9. The column was developed with a 100 ml gradient of the same buffer from 50 to 400 mM. Filled circles represent absorbance at 280 nm, while open circles are absorbance values at 436 nm.