Interactions of Cibacron Blue F3GA and Nucleotides with *Escherichia coli* Aspartate Carbamoyltransferase and Its Subunits†

Inda Issaly, Maryse Poiret, Patrick Tauc, Lucile Thiry, and Guy Hervé*

ABSTRACT: The interaction of two dyes, Cibacron Blue I and Cibacron Blue II (purified from Cibacron Blue F3GA), with native *Escherichia coli* aspartate carbamoyltransferase and its isolated subunits was investigated by kinetic measurements, competition assays, and difference spectroscopy, in the absence or presence of the physiological ligands of this enzyme. At the same time, the influence of adenosine and cytidine phosphate derivatives on the activity of the isolated catalytic subunits was studied. The dyes bind on the native enzyme to the catalytic and regulatory sites, inhibiting the catalytic activity (K_i of Cibacron Blue I = 10 μM) and the action of the effectors ATP and CTP. However, at low concentrations, Cibacron Blue II stimulates slightly the activity. Cibacron Blue I and Cibacron Blue II inhibit the activity of the isolated catalytic subunits. This inhibition is noncompetitive with regard to carbamoyl phosphate at low dye concentrations and becomes mixed at high concentrations. In all cases, this inhibition is noncompetitive with respect to aspartate. Under the same conditions, the adenosine and cytidine phosphate derivatives provoke competitive inhibitions at low concentrations and mixed inhibitions at high concentrations. These inhibitions result from the binding of both dyes and nucleotides to the catalytic site and to a "secondary site". The dyes have a higher affinity for the secondary site than for the catalytic site. Conversely, the nucleotides have a higher affinity for the catalytic site than for the secondary site. This secondary site seems to be masked when catalytic and regulatory subunits are associated. Moreover, an additional CTP binding site seems to be present on the catalytic moiety of the native enzyme. In the presence of dyes or nucleotides (ADP and ATP), some interactions between monomers in the catalytic subunits are detected. The dyes and the nucleotides bind competitively to the isolated regulatory subunits (K_d of Cibacron Blue II = 4 μM), manifesting interactions between the regulatory sites on these subunits. Cibacron Blue thus appears to be a useful probe for studying the catalytic and regulatory site of aspartate carbamoyltransferase by difference spectroscopy.

The hexameric enzyme aspartate carbamoyltransferase from *Escherichia coli* (carbamoylphosphate:L-aspartate carbamoyltransferase, EC 2.1.3.2), made up of two catalytic trimers in contact and bridged by three regulatory dimers, contributes substantially to the control of the pyrimidine pathway by exhibiting the different kinds of regulatory interactions characteristic of allosteric enzymes: homotropic cooperative interactions between the catalytic sites for the binding of the substrate aspartate, heterotropic negative and positive interactions between regulatory and catalytic sites, allowing for inhibition by CTP and stimulation by ATP, respectively, and heterogeneity of CTP and ATP binding sites, which might correspond to homotropic negative cooperativity.

Therefore, this protein has attracted wide interest as a model allosteric enzyme, and much work has been performed in an effort to deduce its structure and to understand the molecular mechanisms of its allosteric behavior. This considerable information has been extensively reviewed (Gerhart, 1970; Jacobson & Stark, 1973a; Thiry & Hervé, 1978; Kantrowitz et al., 1980a,b).

Apparently the essential mechanism of the aspartate carbamoyltransferase (ATCase)[1] regulatory system can be explained by the coupling of a concerted conformational change corresponding to the homotropic interactions between the catalytic sites with local conformational changes resulting from the effectors binding (Thiry & Hervé, 1978). Thus, it is essential to study the molecular mechanisms of that coupling in particular with respect to the action of the nucleotide effectors ATP and CTP.

For the elucidation of the mechanism of these nucleotide interactions, it is necessary to know the nature and the number of the nucleotide effector binding sites on the native enzyme. With regard to the number of sites, numerous investigations (Changeux et al., 1968; Hammes et al., 1970; Winlund & Chamberlin, 1970; Buckman, 1970; Cook, 1972; Winlund-Gray et al., 1973; Matsumoto & Hammes, 1973; Tondre & Hammes, 1974; Allewell et al., 1975; Suter & Rosenbusch, 1977) have yielded divergent results as have the studies of CTP binding to the isolated regulatory subunits (Changeux et al., 1968; Hammes et al., 1970; Suter & Rosenbusch, 1977). The complexity of the binding isotherms is, at least partially, due to the fact that nucleotides also interact with the carbamoyl phosphate site of the enzyme as competitive inhibitors with regard to this substrate (Porter et al., 1969). They probably also bind to another site on the catalytic subunits as was suggested by the experiments of inhibition by adenosine nucleotides (Thiry & Hervé, 1978). Moreover, Monaco et al. (1978) have shown, by X-ray diffraction studies, a binding site for CTP on the catalytic subunit structure.

Most of the results previously reported postulated that ATP and CTP bind to the same site on the regulatory subunits (Gerhart & Pardee, 1962, 1964; Changeux et al., 1968; London & Schmidt, 1972, 1974; Jacobsberg et al., 1975; Baron et al., 1979). Recent studies by Honzatko et al. (1979) show that such is the case.

It is known that Cibacron Blue F3GA, a sulfonated polyaromatic blue dye, can be used for probing nucleotide binding

† From the Laboratoire d'Enzymologie du Centre National de la Recherche Scientifique, 91190 Gif-sur-Yvette, France. *Received May 22, 1981.* This research was supported by the Centre National de la Recherche Scientifique, by the Association pour le Développement de la Recherche sur le Cancer, Villejuif (Contrat No. 6088), by the Délégation Générale à la Recherche Scientifique et Technique (Contrat No. 79.7.0156), by the Direction des Recherches, Etudes et Techniques (Contrat No. 81.1046), and by a fellowship from La Fondation Joliot Curie (Saclay) to I.I. and a grant from U.N.E.S.C.O. (Paris) to I.I.

[1] Abbreviations: ATCase, aspartate carbamoyltransferase; CBI, Cibacron Blue I; CBII, Cibacron Blue II; 2-thioU-ATCase, modified aspartate carbamoyltransferase obtained in the presence of 2-thiouracil.

0006-2960/82/0421-1612$01.25/0

sites in numerous proteins (Wilson, 1976; Thompson & Stellwagen, 1976; Apps & Gleed, 1976; Stellwagen, 1977; Schuber & Pascal, 1977; Kumar & Krakow, 1977; Edwards & Woody, 1977; Grazi et al., 1978; Ashton & Polya, 1978; Beissner & Rudolph, 1978; Nissler et al., 1979; Toste & Cooke, 1979; Moe & Piszkiewicz, 1979; Land & Byfield, 1979; Biellmann et al., 1979; Witt & Roskoski, 1980; Pompon et al., 1980). In the course of the present work, we used the properties of Cibacron Blue F3GA in kinetic measurements, competition assays, and absorption difference spectroscopy to obtain information concerning the binding of the nucleotide effectors to the allosteric and catalytic sites of ATCase. At the same time, the influence of the adenosine and cytidine derivatives on the activity of the isolated catalytic subunits has been investigated.

The results presented provide some insight into the nature of the binding of ATP and CTP on the catalytic subunits and give additional evidence that both nucleotides bind to the same site on the regulatory subunits. Moreover, it is concluded that interactions occur between carbamoyl phosphate sites within the catalytic subunits in the presence of the dyes studied.

Materials and Methods

Chemicals. Lithium carbamoyl phosphate, L-aspartate, AMP, ADP, and ATP were purchased from Sigma; CMP, CDP, and CTP were from P-L Biochemicals; activated silica gel thin-layer chromatography plates (no. 6061) were from Eastman-Kodak; silica gel 7734 was from Merck; [^{14}C]aspartate was from Service de Biochimie, CEN-Saclay, France. Cibacron Blue F3GA (color index name Reactive Blue 2, CI 61211) was a generous gift from Ciba-Geigy and also was purchased from Pierce Chemical Co. All other chemicals were of analytical reagent grade. Nucleotide solutions were controlled by UV absorption spectroscopy and thin-layer chromatography (Beck & Howlett, 1977).

Cibacron Blue F3GA, from Ciba-Geigy, was purified by preparative column chromatography on silica gel (Weber et al., 1979). The fractions were assayed by thin-layer chromatography on activated silica gel developed by tetrahydrofuran–water (48:7 v/v) (Weber et al., 1979). Two major blue compounds, CBI, R_f 0.77, and CBII, R_f 0.55, were obtained. The fractions corresponding to each of these products were pooled, the organic solvents were removed by rotary evaporation, and the aqueous dye solutions were lyophilized and stored at 0–4 °C under vacuum. Under these conditions, CBI appears to be a pure, stable compound, while CBII still contains traces of two contaminants. This product is unstable, and when analyzed by thin-layer chromatography, after storage for several months, it shows four compounds which correspond to some of those observed in the crude dye preparation. The commercial compound from Ciba-Geigy contained about 80% of CBI and 5% of CBII. CBI was also the major compound of the Pierce dye preparation which was almost devoid of CBII. Thus, CBI must correspond to the product the structure of which was already described (Böhme et al., 1972). Preliminary analysis suggests that CBII differs from CBI by the absence of one sulfonated group.

The concentration of solutions of both purified compounds was determined spectrophotometrically (ϵ_{610} = 13.6 mM^{-1} cm^{-1}) (Thompson & Stellwagen, 1976). Absorption spectra were measured with a Cary Model 118 spectrophotometer.

Difference Spectroscopy. Difference spectra of CBI or CBII catalytic or regulatory subunit complexes in the presence or absence of various ligands were recorded at 22 °C with the Cary Model 118 spectrophotometer mentioned above by using tandem match cells. The catalytic subunits were previously dialyzed against 40 mM Tris–acetate (pH 8) containing 10 mM 2-mercaptoethanol and 0.2 mM EDTA. Regulatory subunits were dialyzed against 10 mM imidazole-HCl (pH 7.0) containing 10 mM 2-mercaptoethanol. The spectra were determined at catalytic or regulatory subunit concentrations of about 5 or 10 μM, respectively, or as otherwise indicated. For the base line, identical volumes of the protein solution and of the corresponding buffer were placed in the sample and in the reference cells, and the difference absorbance was measured. Identical volume increments of a concentrated solution of CBI or CBII were added to both cells in the proper compartments, and the difference absorbance was recorded after each addition. When other ligands were studied, the base line was made with identical volumes of dye and buffer solutions.

Enzyme Preparation. ATCase was purified and dissociated into its subunits according to Gerhart & Holoubek (1967) from the special strain of *E. coli* generously provided by Dr. J. Gerhart (University of California, Berkeley). 2-thioU-ATCase was prepared and purified as described elsewhere (Kerbiriou & Hervé, 1972). These different proteins were controlled by electrophoresis in polyacrylamide gels according to Perbal & Hervé (1972).

Enzymatic Assay. The ATCase activity was determined by the method of Porter et al. (1969) under the conditions previously described by Perbal & Hervé (1972) in the presence of 50 mM Tris–acetate (pH 8), 5 mM carbamoyl phosphate, and 5 mM aspartate or as otherwise indicated. The preparations of carbamoyl phosphate used were controlled by incubating an aliquot for 30 min at 37 °C in the presence of 40 μg of catalytic subunits of *E. coli* ATCase and 20 mM [^{14}C]aspartate and deducting the amount of carbamoyl phosphate used from the amount of [^{14}C]carbamoyl aspartate formed under these conditions. The unit of enzyme activity is defined as the amount which catalyzes the formation of 1 μmol of carbamoyl aspartate/h under the conditions of the assay. Specific enzymatic activities are expressed in units per milligram of protein.

Enzyme concentrations were determined either by absorbance measurements at 280 nm using extinction coefficients of 0.59, 0.73, and 0.37 (mg/mL)$^{-1}$ cm^{-1} for native ATCase and catalytic and regulatory subunits, respectively (Kerbiriou et al., 1977), or by the method of Lowry et al. (1951) using bovine serum albumin as a standard, and taking in account the 20% overestimate which is given by this method (Kerbiriou et al., 1977).

The data from the experiments reported in section B (ii and iii) were analyzed by utilizing a linear regression program introduced in a Hewlett-Packard Model 9825 A electronic computer, equipped with a curve plotter.

The experimental results of the competition assays were compared with the values obtained by using the equations proposed by Webb (1963):

$$i_{1,2} < i_1 + i_2 - i_1 i_2 \quad \text{(antagonism)}$$

$$i_{1,2} = i_1 + i_2 - i_1 i_2 \quad \text{(addition)}$$

where i_1 is the inhibition of the catalytic activity in the presence of one inhibitor, i_2 is the inhibition of the catalytic activity in the presence of the second inhibitor, and $i_{1,2}$ is the inhibition of the catalytic activity in the presence of both inhibitors.

Results

(A) Interaction of Cibacron Blue I and II with Native ATCase. (i) Effect of CBI and CBII on the Catalytic Activity. The effects of the purified dyes CBI and CBII on the native ATCase appear to be quite different. CBI is a strong inhibitor

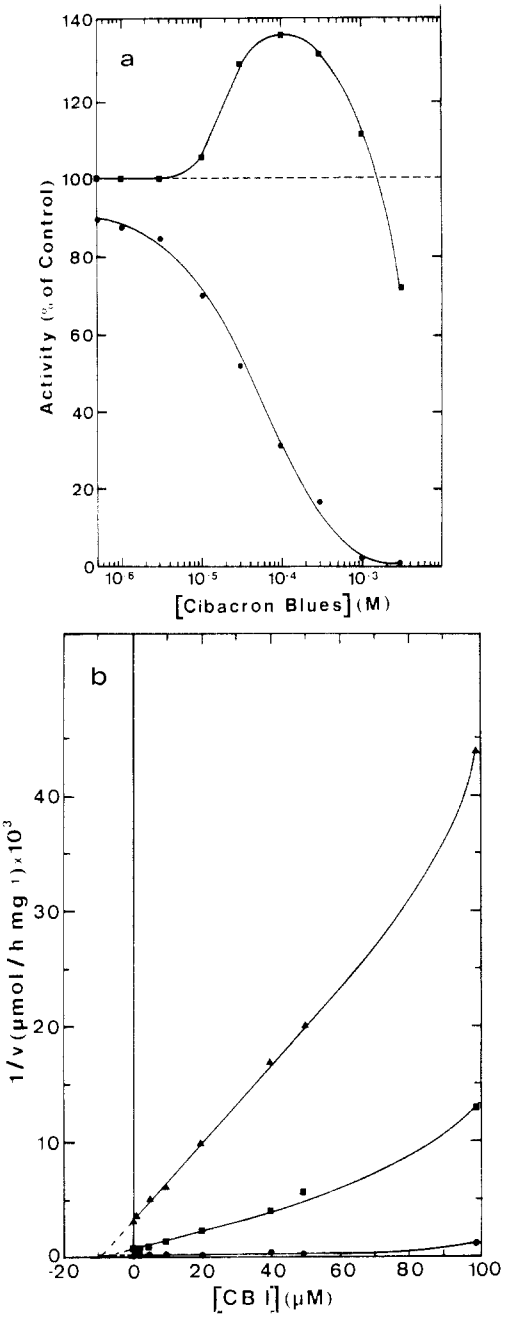

FIGURE 1: Effect of CBI and CBII on the catalytic activity of ATCase. ATCase (0.24 μg) was incubated as indicated under Materials and Methods in the presence of increasing amounts of both dyes. Activity is reported as the percent of activity of enzyme without dyes expressed by the value of 100. (a) Effect of CBI (●); effect of CBII (■). (b) Noncompetitive inhibition vs. aspartate concentration, by CBI. ATCase (0.32 μg) was incubated as indicated under Materials and Methods in the presence of increasing amounts of CBI and various aspartate concentrations. The results are presented according to the Dixon plot: 1 (▲), 3 (■), and 20 mM aspartate (●).

of the activity; an inhibition of 12% is already detectable at 1 μM. This inhibition increases with dye concentration up to 98% in the presence of 1 mM CBI (Figure 1a). The dependence of activity of the native enzyme on CBI concentration was analyzed by Dixon plots (Dixon, 1953) of the reciprocal activity vs. CBI concentration in the presence of 1.3 and 20 mM aspartate (Figure 1b). At low CBI concentrations, the inhibition is noncompetitive with respect to aspartate, and an extrapolation of the Dixon plots yields a K_i of approximately 10 μM. However, when CBI concentrations are higher than 0.5 mM, upward curvatures are obtained, indicating more complex inhibition.

Table I: Effect of CBI on the Feedback Inhibition of ATCase Activity by CTP

[CBI] (mM)	$R = i_{1,2} - i_1 - i_2 + i_1 i_2$[a] at 0.5 mM CTP
0.001	−2.5
0.003	−8.6
0.010	−4.0
0.030	−2.6
0.100	−1.3
0.300	−1.6

[a] According to the equation proposed by Webb (1963); see Materials and Methods.

In contrast, at concentrations of CBII lower than 1 mM, activation of the enzyme is observed (Figure 1a). Since this dye behaves usually as an ATP analogue, this result suggests that it might bind to the regulatory site and mimick the ATP stimulatory effect. This interpretation is consistent with the partial reversion of the sigmoidal shape of the aspartate saturation curve, the Hill number decreasing from 1.8 to 1.6 in the presence of 0.1 mM CBII. At higher concentrations of the dye, an inhibition of the catalytic activity is observed (Figure 1a).

It has been reported that Cibacron Blue or one of its degradation products is able to react covalently with proteins (Weber et al., 1979). It was verified that in the present case such a reaction is very slow and cannot be significant in the time course of the experiments performed.

(ii) Effects of CBI and CBII on the Stimulation by ATP and the Feedback Inhibition by CTP. If both dyes and nucleotide effectors bind to the regulatory sites on the enzyme, it should be possible to completely hinder ATP activation and CTP inhibition of the native enzyme by the addition of CBI and CBII. Moreover, in such a case, if the slight activation provoked by CBII results from its binding to these regulatory sites, it should not be additive to the stimulatory effect of ATP.

Figure 2a shows the effects of CBI and CBII (with or without ATP) on the activity of the native ATCase, and Figure 2b shows the calculated intrinsic influence of these dyes on the stimulation of the enzyme activity by ATP, deducting their net effect on catalysis. Indeed, it appears that the activation of the enzyme by ATP is totally abolished by increasing amounts of CBI and CBII from 0.03 up to 1 mM. Moreover, these figures show also that the stimulations provoked by 1 mM ATP and CBII (from 0.03 up to 1 mM) are not additive (Figure 2a). In both cases, at low concentrations of CBI and CBII, a slight increase of the activation by ATP is also observed (Figure 2b).

The effects of CBI and CBII (with or without CTP) on the activity of the native enzyme are presented in Figure 3a. Using the different values reported in this figure for CTP and CBI inhibitions in the calculation of Webb (1963), it can be seen that the inhibitions provoked by these two ligands are not additive (Table I). Figure 3b shows the calculated intrinsic influence of the dyes on the inhibition of the enzyme activity by CTP, deducting their net effect on catalysis. It appears that CBI abolishes the inhibition by CTP. Such an effect is not observed with CBII for concentrations below 1 mM. This is most probably due to the slight stimulation by CBII reported in section A (i). These results suggested that on the native enzyme CBI and CBII act on the same sites as ATP and CTP.

(B) Interaction of CBI, CBII, and Nucleotides with Isolated Catalytic Subunits. The effect of the dyes on the native enzyme must be the consequence of their binding to its catalytic and/or regulatory moieties. The behavior of both compounds toward the interactions of substrates and nucleo-

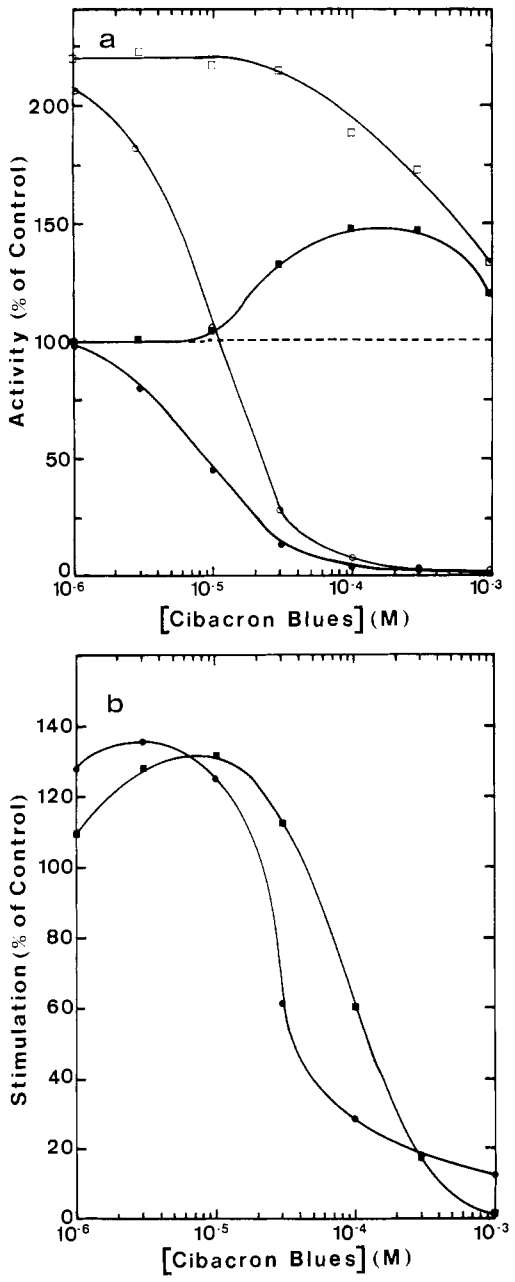

FIGURE 2: Effect of CBI and CBII on the stimulation of ATCase activity by ATP. ATCase (0.24 μg) activity was determined as indicated under Materials and Methods, without and with ATP, in the presence of increasing amounts of CBI or CBII. (a) Effect of CBI and CBII concentration without and with ATP on the catalytic activity. Activity is reported as indicated in Figure 1. Inhibition by CBI (●); stimulation by CBII (■); effect of CBI *plus* 5 mM ATP (○); stimulation by CBII *plus* 1 mM ATP (□). (b) Effect of CBI and CBII concentration on the stimulation by ATP. The data were calculated by deducting the net effect of the dyes on the catalytic activity in the absence of ATP shown in (a). Effect of CBI on the stimulation by ATP (■); effect of CBII on the stimulation by ATP (●).

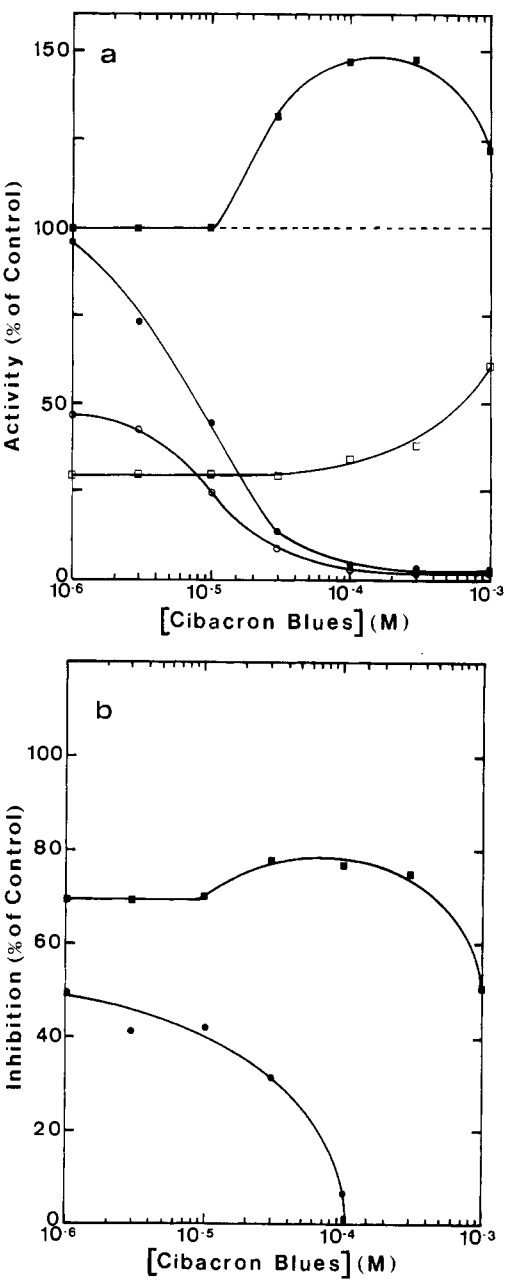

FIGURE 3: Effect of CBI and CBII on the feedback inhibition of ATCase activity by CTP. ATCase (0.24 μg) activity was assayed in the presence of 10 mM carbamoyl phosphate in the experiments with CBI. Other conditions as described under Materials and Methods. Incubations were performed, without and with CTP, in the presence of increasing amounts of CBI or CBII. (a) Effect of CBI and CBII without and with CTP on the catalytic activity. Activity is reported as indicated in Figure 1. Inhibition by CBI (●). Stimulation by CBII (■). Inhibition by CBI *plus* 0.5 mM CTP (○). The inhibition of the catalytic activity by 0.5 mM CTP was 53.2% of the control value. Inhibition by CBII *plus* 1.0 mM CTP (□). The inhibition of the catalytic activity by 1 mM CTP was 72% of the control values. (b) Effect of CBI and CBII on the feedback inhibition by CTP. The data were calculated by deducting the net effect of the dyes on the catalytic activity in the absence of CTP, shown in (a). Effect of CBI on the feedback inhibition by CTP (●). Effect of CBII on the feedback inhibition by CTP (■).

tides with the catalytic subunits was studied by steady-state kinetics, competition experiments, and difference spectroscopy. On this occasion, the influence of the different adenosine and cytidine phosphate derivatives on these subunits was also investigated.

(*i*) *Effects of CBI and CBII on the Steady-State Kinetics.* Catalytic subunit activity is inhibited by CBI and CBII (Figure 4). A comparison of Figures 1 and 4 shows that native ATCase is more sensitive than catalytic subunits to the inhibition by CBI, suggesting again that part of the inhibition of the native enzyme by this dye is due to its binding to the regulatory subunits. CBII is only an inhibitor of catalytic subunit activity, indicating that its stimulatory effect on the native ATCase is also the result of its binding to the regulatory subunits.

The shape of the inhibition curve of catalytic subunits at increasing concentrations of both dyes is not hyperbolic, suggesting either a mixed effect or a phenomenon of coop-

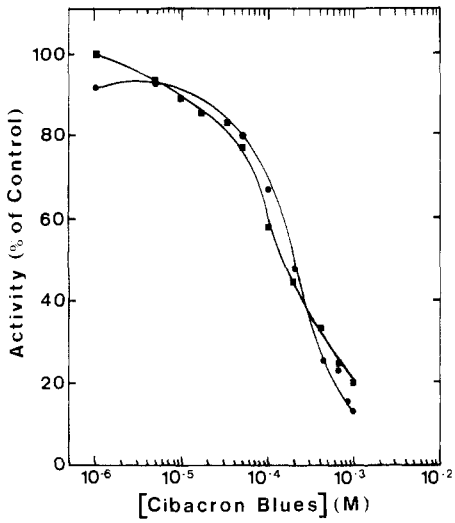

FIGURE 4: Inhibition of isolated catalytic subunit activity by CBI and CBII. Catalytic subunit (0.234 μg) activity was tested as indicated under Materials and Methods in the presence of increasing amounts of both dyes. Activity is reported as in Figure 1. Inhibition by CBI (●) and inhibition by CBII (■).

Table II: Kinetic Parameters for Isolated Catalytic Subunits in the Presence of CBI and CBII[a]

[CBI] (mM)	[CBII] (mM)	K_m (mM)	$S_{0.5}$ (mM)	V_{max} [μmol h^{-1} (mg of protein)$^{-1}$]	Hill no.
0		0.90		10000	
0.10			1.12	6666	1.6
0.50			2.24	2857	1.8
	0	0.71		10000	1.0
	0.10		1.12	5000	1.1
	0.50		1.41	2000	1.4

[a] Data from Figure 5a,b. K_m and $S_{0.5}$ for carbamoyl phosphate. $S_{0.5}$ values were calculated from the corresponding Hill plots. The maximum velocities were estimated by extrapolation of the Lineweaver-Burk double-reciprocal plots.

erativity. The concentrations of CBI and CBII which give half-maximal inhibition, $I_{0.5}$, are 0.15 and 0.075 mM, respectively.

Both dyes convert the hyperbolic saturation curve for carbamoyl phosphate into a sigmoidal one. Consequently, the corresponding Lineweaver–Burk double-reciprocal plots are distinctly nonlinear, showing upward curvatures (Figure 5a,b). Thus, it seems that both dyes are able to induce apparent homotropic positive interactions between carbamoyl phosphate binding sites. The different values of K_m, $S_{0.5}$, V_{max}, and Hill numbers which were calculated are presented in Table II. These results indicate that both dyes bind to a high-affinity site which is distinct from the catalytic one.

The double-reciprocal plots of the velocity of the catalytic subunits' activity vs. aspartate concentration in the presence of CBI show a pattern of noncompetitive inhibition with respect to this substrate. According to the Dixon plot, the K_i is 0.22 mM. CBII is also a noncompetitive inhibitor of the enzyme with regard to aspartate, with a K_i of 0.27 mM. The fact that CBI and CBII appear not to compete with aspartate is consistent with the ordered mechanism of substrate binding (Porter et al., 1969; Collins & Stark, 1969, 1971; Hammes et al., 1971; Schaffer & Stark, 1972; Wedler & Gasser, 1974).

It is noteworthy that CBI and CBII have a very similar behavior toward the isolated catalytic subunits, in contrast with the quite different influences they have on the native enzyme.

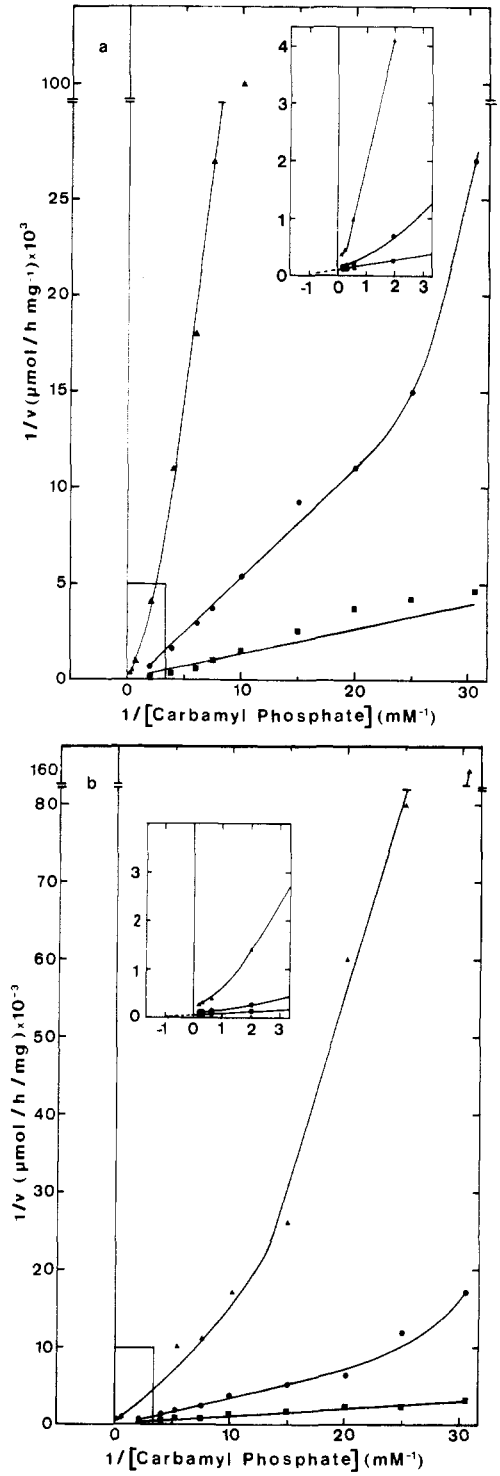

FIGURE 5: Noncompetitive and mixed inhibition by CBI and CBII vs. carbamoyl phosphate concentration of isolated catalytic subunits. Catalytic subunits (0.15 μg) were incubated as indicated under Materials and Methods in the presence of increasing amounts of carbamoyl phosphate, without and with various concentrations of CBI or CBII. (a) Lineweaver–Burk double-reciprocal plot of velocity vs. carbamoyl phosphate concentration without (■) and with 0.1 (●) and 0.5 mM CBI (■). (b) Lineweaver–Burk double-reciprocal plot of velocity vs. carbamoyl phosphate concentration without (■) and with 0.1 (●) and 0.5 mM CBII (▲).

(ii) Effects of Adenosine and Cytidine Phosphate Derivatives. It is known that phosphate ions as well as the nucleoside triphosphate CTP are competitive inhibitors of carbamoyl phosphate in the active sites of the native enzyme and catalytic subunits (Kleppe, 1966; Porter et al., 1969). In addition, it has been shown that at high concentrations, ATP, ADP, and

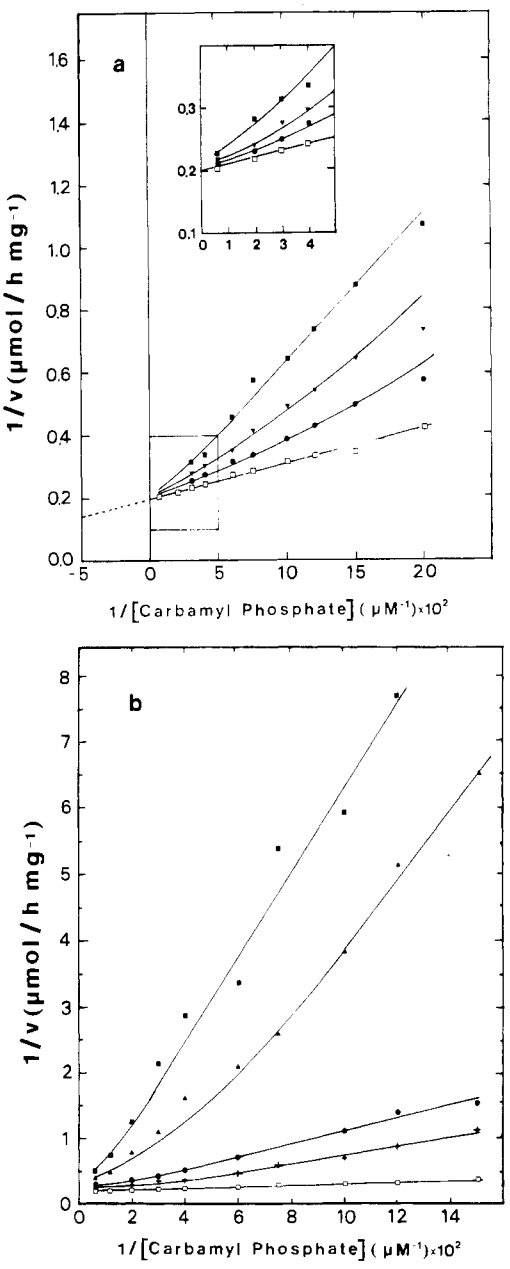

FIGURE 6: Mixed inhibition vs. carbamoyl phosphate concentration of isolated catalytic subunits by adenosine phosphate derivatives. Catalytic subunit (0.04–0.09 µg) activity was tested in the presence of 3 µM aspartate (specific radioactivity of 0.4 µCi/nmol) and increasing amounts of carbamoyl phosphate, without and with various concentrations of adenine nucleotides. Other conditions as described under Materials and Methods. The results are presented according to the Lineweaver–Burk double-reciprocal plot. (a) Mixed inhibition by ADP without (□) and with 0.05 (●), 0.1 (▼), and 0.2 mM ADP (■). (b) Mixed inhibition by ATP without (□) and with 0.1 (+), 0.2 (●), 0.66 (▲), and 1.33 mM ATP (■).

AMP inhibit the catalytic subunit activity, whatever the number of phosphate groups bound to adenosine (Thiry & Hervé, 1978). Furthermore, the results reported above [section B (i)] indicate that the inhibition of the catalytic subunit activity by Cibacron Blues results partially from their binding to a site which is distinct from the catalytic one. Since these dyes often act as nucleotide analogues, the possibility that ATP and CTP bind to this additional site on the isolated catalytic subunits was considered. Consequently, the influence of the different adenosine and cytidine phosphate derivatives on the activity of the isolated catalytic subunits has been more extensively investigated.

(*1*) *Adenosine Derivatives.* The double-reciprocal plots of the velocity of the catalytic subunit activity vs. carbamoyl phosphate concentration in the presence of increasing amounts of AMP (from 1 up to 10 mM) are linear and clearly exhibit both competitive and noncompetitive inhibition, confirming that this nucleotide binds to the catalytic site and to a secondary site. Competitive binding predominates with a calculated K_i of 1.42 mM (Dixon, 1964); noncompetitive binding is significantly weaker with a K_i of 11.5 mM.

Table III: Effect of ATP on the Inhibition of Isolated Subunits by CTP[a]

[ATP] (mM)	$R = i_{1,2} - i_1 - i_2 + i_1 i_2$ 5 mM CTP	10 mM CTP
1		−1.9
5	−8.5	−4.0
10	−5.1	−8.5
50	−5.0	−6.0

[a] Catalytic subunit (0.145 µg) activity was tested under the conditions described under Materials and Methods. R is defined as indicated in Table I.

The double-reciprocal plots of the velocity of catalytic subunit activity vs. carbamoyl phosphate concentration in the presence of increasing amounts of ADP and ATP show a more complex pattern (Figure 6a,b) and suggest a mixed inhibition as in the case of Cibacron Blues. The $I_{0.5}$ values for these two compounds are 9 and 6 mM, respectively. It is interesting to note that, in these cases, the double-reciprocal plots are nonlinear, indicating that, like the Cibacron Blues, ATP and ADP induce apparent cooperativity in carbamoyl phosphate utilization. The different values of K_i and $I_{0.5}$ for these nucleotides show that the inhibition of catalytic subunits by adenosine nucleotides increases as a function of the number of phosphate groups bound to the nucleoside.

Only carbamoyl phosphate has an effect on the inhibition of the catalytic activity by ATP, and this inhibition is not dependent on the aspartate concentration. Again, this fact is consistent with the ordered mechanism of substrate binding (Porter et al., 1969; Collins & Stark, 1969, 1971; Hammes et al., 1971; Schaffer & Stark, 1972; Wedler & Gasser, 1974).

(*2*) *Cytidine Derivatives.* The double-reciprocal plots of the velocity of the catalytic subunit activity vs. carbamoyl phosphate concentration in the presence of increasing amounts of CMP, CDP, and CTP are linear and exhibit both competitive and noncompetitive inhibition. In all cases, competitive binding predominates with calculated K_i values of 2.2, 0.16, and 0.11 mM (Dixon, 1964), respectively, while noncompetitive binding is significantly weaker with calculated K_i values of 22, 6, and 2.1 mM, respectively. Consequently, it appears that the different cytidine derivatives bind to the catalytic site and to a secondary site.

A comparison of these nucleotide K_i values clearly shows that their affinity for the secondary binding site increases with the number of phosphate groups. Their effect on the K_m of carbamoyl phosphate is certainly due to the competition for the catalytic site between the nucleotidic phosphate groups and this substrate (Porter et al., 1969). In this case, the affinity of these compounds for the catalytic binding site is less dependent on the number of phosphate groups, since CDP and CTP have a similar K_i, only CMP showing a lower affinity.

The possibility that the secondary binding site is the same for adenosine and cytidine derivatives is supported by the fact that the inhibitions produced by 5, 10, or 50 mM ATP and 5 or 10 mM CTP, at saturating concentration of carbamoyl phosphate, are not additive (Table III), suggesting the competition of both effectors for the same site.

Table IV: Effect of CBI and CBII on the Inhibition of Isolated Catalytic Subunits by CTP and ATP[a]

	$R = i_{1,2} - i_1 - i_{2\,2} + i_1 i_2$		
	10 mM CTP	20 mM CTP	3.66 mM ATP
[CBI] (mM)			
0.033	−3.0	+1.0	
0.050	−5.2	−2.9	
0.100	−5.0	−2.5	−10
0.200	−4.0	−1.0	
0.330			−10
0.400	−1.4	+0.6	
0.500			−4
0.660		−1.0	−8
[CBII] (mM)			
0.05	+0.4		
0.100	+0.1	−3.4	−22
0.200	−1.2	−2.3	
0.330			−8
0.440	−1	−0.9	
0.500			−11
0.660	−1.5	−1.5	

[a] Catalytic subunit (0.15 μg) activity was determined as described under Materials and Methods. R is defined as indicated in Table I.

With regard to the mixed inhibition observed in the case of AMP and the cytosine derivatives, the computerized method used (cf. Materials and Methods) gave the same result in three different experiments for each nucleotide, and the quality and significance of the fitted parameters are illustrated in the next section where only competitive inhibition is observed.

(iii) Effect of AMP on the Catalytic Activity of 2-ThioU-ATCase. It was interesting to determine if the secondary nucleotide binding site of catalytic subunits is accessible or not in the native enzyme. For this purpose, the effect of AMP on the catalytic activity of 2-thioU-ATCase was examined. This modified enzyme, in which the homotropic cooperative interactions between catalytic sites are selectively abolished (Kerbiriou & Hervé, 1972, 1973; Kantrowitz et al., 1977; Kerbiriou et al., 1977), was used in order to avoid the interference of these interactions with the effect of the nucleotides. Moreover, AMP was used, instead of ATP, because of its much lower influence on the catalytic activity through their binding to the regulatory sites (Gerhart & Pardee, 1962; Thiry & Hervé, 1978). AMP (from 1 up to 10 mM) provokes strictly competitive inhibition of the activity of 2-thioU-ATCase, indicating that when catalytic and regulatory subunits are associated in the native enzyme the secondary nucleotide binding site of the catalytic subunits is masked.

(iv) Effect of CBI and CBII on the Inhibition by ATP and CTP. If, as suggested in the case of the regulatory sites, both the dyes and the nucleotide effectors were to bind to the same sites on the catalytic subunits, the inhibitions by ATP or CTP and CBI or CBII should not be additive. Taking into account the competition assays of ATP or CTP and both dyes on the native enzyme described above, we performed similar experiments with these compounds by using the isolated catalytic subunits.

Table IV shows that, indeed, the inhibitions provoked by 3.66 mM ATP and CBI or CBII (from 0.10 up to 0.66 mM) are not additive (Webb, 1963). These data indicate that ATP and both dyes bind to the same sites on the isolated catalytic subunits as was the case for the native enzyme.

Similar results were obtained for the inhibition of isolated catalytic subunits by 10 mM CTP and increasing concentrations of CBI from 0.033 up to 0.66 mM; these inhibitions are not additive (Table IV). On the contrary, when the concentration of CTP is raised to 20 mM, the inhibitions provoked by this nucleotide and CBI (from 0.033 up to 0.66 mM) become additive (Table IV) according to Webb (1963). These facts suggest that, at low concentrations, CTP binds to the same site as CBI and that it binds also to another site when present at high concentrations.

The results for CBII presented in Table IV show that the inhibitions provoked by 10 mM CTP and increasing concentrations of CBII from 0.05 up to 0.66 mM are additive (Webb, 1963). Thus, it appears that also in this case CTP is able to bind to a site which is distinct from the CBII binding site. On the other hand, the inhibitions provoked by 20 mM CTP and increasing amounts of CBII from 0.10 up to 0.66 mM are not additive, suggesting the binding of CTP to the same site as the dye.

The results reported in this section indicate that ATP and CTP bind, on the isolated catalytic subunits, to the same sites as both dyes. Furthermore, it seems that CTP binds to two different sites: one of these sites binds CBI; the other one binds CBII at the dye concentrations used in these experiments.

(v) Difference Spectra of CBI and CBII in the Presence of Isolated Catalytic Subunits and Effects of the Ligands. As previously mentioned, Cibacron Blue F3GA has been postulated to interact at the nucleotide sites of the nucleotide-requiring enzymes (Wilson, 1976), as a consequence of a structural similarity between this dye and the nucleotides (Böhme et al., 1972; Thompson et al., 1975; Stellwagen, 1977), as well as those proteins possessing a "dinucleotide fold" (Thompson et al., 1975; Thompson & Stellwagen, 1976; Stellwagen, 1977). The binding of the dye at the nucleoside phosphate site produces a red shift in its absorption spectrum (Thompson & Stellwagen, 1976). Therefore, we expected similar behavior of CBI and CBII when binding to ATCase and its subunits. Furthermore, with the aim of confirming the interpretations of the results reported above, we studied the influence of the substrate carbamoyl phosphate and of the effectors ATP and CTP on the difference spectra of these dyes in the presence of the catalytic subunits.

(1) Difference Spectra Produced When CBI and CBII Bind to the Isolated Catalytic Subunits. In aqueous solution, CBI exhibits a spectral absorption maximum at 610 nm. The maximum is at 600 nm in the case of CBII. In the presence of 5 μM catalytic subunits, the absorption spectrum of both dyes undergoes a red shift, producing a difference spectrum showing a maximum and a minimum centered at 668 and 580 nm, respectively (Figure 7). The absence of an isosbestic point after successive additions of the dyes to the enzyme indicates that more than one type of complex is formed. Upon saturation of catalytic subunits with CBI, the maximum effect at 668 nm is obtained when the effect at 580 nm is still increasing, whereas the contrary is observed in the case of CBII. Such a phenomenon was already reported in the case of the binding of Cibacron Blue F3GA to cytochrome b_5 reductase (Pompon et al., 1980).

These results suggest that the dyes bind to two distinct sites with different affinities, one dye binding more tightly to the site at which the other one binds less tightly. This interpretation is consistent with the results of the competition experiments reported in section B (iv). The determinations of the corresponding dissociation constants are not possible because of the absence of an isosbestic point.

(2) Influence of Carbamoyl Phosphate, ATP, and CTP on the Difference Spectra. If, as was expected, CBI and CBII bind to the carbamoyl phosphate site, and to another site where ATP and CTP also bind, the addition of these compounds to

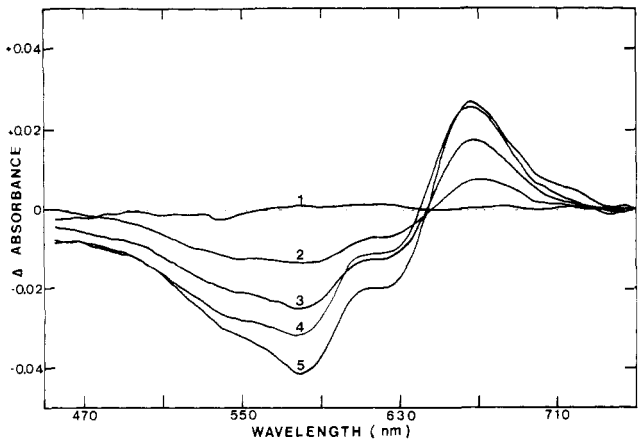

FIGURE 7: Difference spectra of CBI in the presence of isolated catalytic subunits. The difference spectra were determined without Cibacron Blue (1) (base line) and with 9.9 (2), 19.6 (3), 29.1 (4), and 77.3 μM CBI (5), in the presence of 5 μM catalytic subunits, under the conditions indicated under Materials and Methods. The buffer was 40 mM Tris–acetate (pH 8) containing 10 mM 2-mercaptoethanol and 0.2 mM EDTA.

the complexes of the dyes with the catalytic subunits should displace these dyes, resulting in the loss of the observed difference spectra.

As shown in Figure 8a, addition of increasing concentrations of carbamoyl phosphate from 14.8 up to 28.1 mM to the catalytic subunit–CBI complex results in a progressive decrease in the difference absorbance which is more pronounced at 668 nm than at 580 nm. Similar results were obtained in the case of the catalytic subunit–CBII complex except that, in this case, the stronger absorbance decrease is observed at 580 nm. An identical result was obtained in the case of the binding of Cibacron Blue to dihydrofolate reductase (Chambers & Dunlap, 1979). These results indicate that a partial displacement of both dyes is provoked by the addition of carbamoyl phosphate.

The addition of increasing concentrations of ATP to the catalytic subunit–CBI and the catalytic subunit–CBII complexes provokes the progressive abolition of the difference spectra (Figure 8b). Thus, ATP appears to be able to completely displace the two dyes.

Increasing amounts of CTP from 16 to 41.3 mM shift to 700 nm the maximum of the catalytic subunit–CBI complex difference spectrum and, surprisingly, increase substantially its difference absorbance (Figure 8c), indicating that, at the concentrations used, CTP is not able to displace the dye but, on the contrary, it seems either to improve its binding or to alter the environment of its binding site. These results suggest that CTP might also bind to a site which is distinct from the CBI binding site. This interpretation is in accord with the data of the competition experiments reported in section B (iv).

The addition of CTP from 0.8 up to 9.6 mM to the catalytic subunit–CBII complex slightly increases the difference absorbance, and at higher concentrations of this effector, the difference absorbance slightly decreases, indicating that high concentrations of CTP are required to displace partially the dye. These results suggest that, at low concentrations, CTP binds to a site which is distinct from the CBII binding site to which it binds at high concentrations. This interpretation is consistent with the data obtained from the competition assays reported above.

(C) Interaction of CBI, CBII, and Nucleotides with Isolated Regulatory Subunits. From the fact that ATP reverses completely the effect of CTP (Gerhart & Pardee, 1962, 1964; Thiry & Hervé, 1978) and from the pattern for their competitive binding to the native enzyme (Changeux et al., 1968; Jacobsberg et al., 1975), it was interpreted that these nucleotide effectors bind to the same regulatory sites (Changeux et al., 1968; London & Schmidt, 1972, 1974). Moreover, it has been recently demonstrated by X-ray diffraction studies that ATP and CTP bind to the same local region on the native enzyme (Honzatko et al., 1979).

Since the dyes and the nucleotides appear to bind to the same sites on the catalytic subunits, it was interesting to study their possible interactions with the regulatory sites on the isolated regulatory subunits. For this purpose, the difference spectra of CBI and CBII in the presence of isolated regulatory subunits, and the effects of ATP and CTP on these spectra, were investigated.

(i) Difference Spectra Produced When CBI and CBII Bind to the Isolated Regulatory Subunits. In the presence of 10.6 μM regulatory subunits, the absorption spectrum of CBI undergoes a red shift, producing a difference spectrum showing a maximum and a minimum centered at 688 and 588 nm, respectively. The absence of an isosbestic point after successive additions of the dye to the protein indicates that more than one type of complex is formed (Figure 9a) as is the case for the catalytic subunits. Similarly in the presence of 12.7 μM regulatory subunits, the absorption spectrum of CBII undergoes a red shift, producing a difference spectrum showing a maximum and a minimum at 685 and 585 nm, respectively. However, in this case, an isosbestic point at 655 nm is observed, and the increase in the absorbance exhibits a hyperbolic dependence on the concentration of the added dye, indicating that CBII binds to the regulatory subunits on a single type of sites. From this hyperbolic dependence, a dissociation constant of 4 μM and a total of two sites for CBII binding per regulatory subunit (of molecular weight 34 000) were calculated. The presence of an isosbestic point indicates that these two sites interact in the same way with the dye.

(ii) Influence of ATP and CTP on the Difference Spectra. The addition of increasing amounts of ATP (from 8.6 up to 20 mM) or CTP (from 3.5 up to 24 mM) to the regulatory subunit–CBI complex provokes the progressive abolition of the difference spectra (Figure 9b,c), indicating that CBI and the nucleotides bind to the same site. The effects of 16.6 mM CTP and 21.4 mM ATP on the difference absorbance are additive.

The addition of increasing amounts of ATP (from 0.53 up to 15.8 mM) or CTP (from 0.88 up to 4.8 mM) to the regulatory subunit–CBII complex provokes the disappearance of its difference spectra, showing that both nucleotide effectors displace the dye from its binding site, and again the effects of 7.0 mM CTP and 6.8 mM ATP on the difference absorbance are cumulative. However, it is interesting to note that upon successive additions of both nucleotides an isosbestic point is observed no longer. This phenomenon might result from the fact that the binding of the nucleotides on one site of the regulatory dimer would provoke a modification of the environment of the complementary site. This interpretation is consistent with the results of the binding experiments on the native enzyme, showing a heterogeneity of the regulatory sites (Winlund & Chamberlin, 1970; Winlund-Gray et al., 1973; Matsumoto & Hammes, 1973; Allewell et al., 1975) since this heterogeneity is explained in terms of negative cooperativity (Winlund & Chamberlin, 1970; Cook, 1972; Winlund-Gray et al., 1973; Matsumoto & Hammes, 1973; London & Schmidt, 1974; Tondre & Hammes, 1974). However, a direct effect of proximity is not excluded.

Discussion

The results obtained show clearly that Cibacron Blues (CBI

and CBII) bind to ATCase and to both its isolated catalytic and regulatory subunits, in competition with the physiological ligands.

It has been proposed that Cibacron Blue F3GA binds specifically to the so-called "dinucleotide fold", a supersecondary structure involved in the binding of the dinucleotides' enzyme cofactors (Thompson et al., 1975; Thompson & Stellwagen, 1976; Stellwagen, 1977). It was later reported that this dye can also bind to the adenine derivative binding sites on proteins, especially those for ATP (Böhme et al., 1972; Thompson et al., 1975; Apps & Gleed, 1976; Stellwagen, 1977; Beissner & Rudolph, 1978). Finally, recent investigations show that the specificity of Cibacron Blue F3GA is much less restricted (Beissner et al., 1979) and that it also binds to hydrophobic pockets or patches where the active sites of the enzymes are generally located. Such a binding was reported to occur in competition with dihydro- and tetrahydrofolate (Wilson, 1976; Chambers & Dunlap, 1979; Ramesh & Rao, 1980), cyclic AMP (Ashton & Polya, 1978), lactate and flavin (Pompon & Lederer, 1978; Pompon et al., 1980), fructose diphosphate (Grazi et al., 1978), and tRNA (Moe & Piszkiewicz, 1979). This particular location of substrate and cofactor binding sites in hydrophobic regions of proteins has long been noticed (Glazer, 1970). However, it appears that the binding of Cibacron Blue involves both hydrophobic binding and ionic interactions through the sulfonic groups of this dye and that the relative importance of these two effects varies from case to case (Stellwagen, 1977; Beissner & Rudolph, 1978; Land & Byfield, 1979; Edwards & Woody, 1977, 1979). Most probably, these two kinds of interactions are involved in the binding of Cibacron Blues to ATCase and its subunits, considering that all the physiological ligands of this protein are negatively charged.

The experiments reported above give evidence for the existence of at least two binding sites for both dyes and nucleotides on the catalytic subunits, namely, the catalytic site and a "secondary site". CBI and CBII bind to the secondary site, provoking a noncompetitive inhibition of the catalytic activity. Under these conditions, an apparent cooperativity for carbamoyl phosphate utilization can be detected. This phenomenon might result from the fact the binding of Cibacron Blues to the secondary site would slightly impair the entire structure of the catalytic subunits, this impairment being reversed by carbamoyl phosphate binding. Other lines of evidence have indicated that, under certain conditions, carbamoyl phosphate sites within isolated catalytic subunits may not be fully independent and exhibit positive or negative cooperativity (Jacobson & Stark, 1973b; Greenwell et al., 1973; Suter & Rosenbusch, 1976; Lauritzen et al., 1980). Cibacron Blues bind also to the catalytic site in competition with carbamoyl phosphate. Under these conditions, the inhibition remains noncompetitive with regard to aspartate. These results indicate that Cibacron Blues cannot bind to the catalytic site of the carbamoylphosphate–enzyme complex and are consistent with the ordered mechanism of substrate binding (Porter et al., 1969; Collins & Stark, 1969, 1971; Hammes et al., 1971; Schaffer & Stark, 1972; Wedler & Gasser, 1974).

The adenosine and cytidine derivatives appear to have virtually the same effects as Cibacron Blues on the activity of the catalytic subunits. However, these nucleotides have a higher affinity for the carbamoyl phosphate site than for the secondary site. In addition, only ADP and ATP induce the apparent cooperativity for carbamoyl phosphate utilization. A similar effect of ITP has been reported (Heyde, 1973).[2] Furthermore the inhibitions by ATP and CTP are not additive, showing that these two nucleotides bind to the same sites.

The above data and the results of the competition assays in kinetic and difference spectroscopy experiments indicate that both dyes and the nucleotides bind to the catalytic site and to the secondary site. However, the presence of an additional CTP binding site to which CBII binds with a higher affinity than CBI is suggested. This additional site might be the CTP binding site shown by X-ray diffraction studies (Monaco et al., 1978).

The fact that the inhibition provoked by AMP is of a mixed type in the case of the catalytic subunits and only competitive in the case of 2-thioU-ATCase suggests that the secondary nucleotide binding site, which is responsible for the noncompetitive inhibition of the catalytic subunits, is hidden when catalytic and regulatory subunits are associated. The existence of this secondary nucleotide binding site might be of significance concerning the evolution of ATCases. It is well established that some bacteria like *Bacillus subtilis* (Bethell & Jones, 1964; Brabson & Switzer, 1975) possess an ATCase which is only the equivalent of the trimeric catalytic subunits and is not regulated by the nucleotide effectors. Furthermore, it has been shown that wheat germ ATCase is also a trimer of similar molecular weight (R. Yon, personal communication) which is sensitive to feedback inhibition by UMP (Yon, 1972). Thus, the secondary site evidenced in the present work could be a relic of a regulatory site present in an ancestral ATCase devoid of regulatory subunits. Such a view is consistent with the recently proposed scheme for the evolution of ATCase in Enterobacteriaceae (Wild et al., 1980). This hypothesis is supported by the fact that the affinity of the secondary site for the nucleotides increases with the number of phosphate groups present in the nucleotides as is the case of the regulatory sites of native *Escherichia coli* ATCase (Gerhart & Pardee, 1962; Tondre & Hammes, 1974; Thiry & Hervé, 1978; Honzatko et al., 1979). The present low affinity of this secondary site would result from the absence of counterselection of mutationally altered variants of this nowadays useless structure.

Both dyes appear to bind the isolated regulatory subunits, in competition with ATP and CTP on two sites which most likely are the regulatory sites. This behavior is reminiscent of the effect on the ATCase of linear benzo-ATP (Van Derliyn et al., 1978) and tetraiodofluorescein (Jacobsberg et al., 1975), two products which share common structural features with Cibacron Blues. The effect of CBII is particularly similar to that of tetraiodofluorescein which stimulates the ATCase activity at low concentrations and progressively inhibits this activity when its concentration increases (Jacobsberg et al., 1975).

The binding of CBI and CBII to the regulatory sites reveals either a heterogeneity of these sites or some kind of interaction between them. This observation is consistent with the available information concerning these sites (Winlund & Chamberlin, 1970; Cook, 1972; Winlund-Gray et al., 1973; Matsumoto & Hammes, 1973; London & Schmidt, 1974; Tondre & Hammes, 1974; Allewell et al., 1975; Honzatko et al., 1979).

The reported influence of CBI and CBII on the catalytic and regulatory properties of the native enzyme appears to be the logical result of the above-discussed effects of these dyes on the isolated catalytic and regulatory subunits. In conclusion,

[2] It should be noted that this effect of ITP could not be reproduced in our laboratory in spite of the fact that the same conditions were used. Only an uncompetitive inhibition of the catalytic subunit activity by this nucleotide was observed.

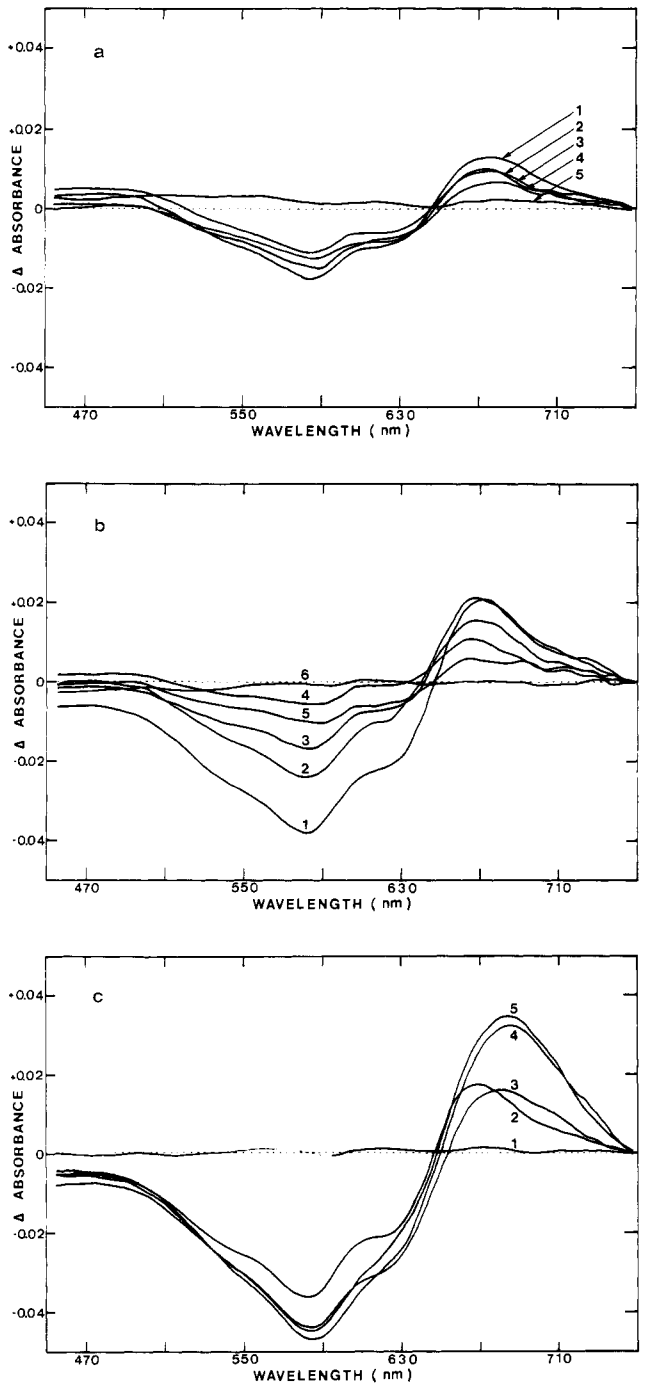

FIGURE 8: Influence of carbamoyl phosphate, ATP, and CTP on the difference spectrum of the isolated catalytic subunit–CBI complex. The difference spectra were recorded before and after the addition of increasing amounts of the indicated ligands to a mixture of 5.2 μM catalytic subunits with 0.10 mM CBI in the buffer indicated in Figure 7 and under the conditions described under Materials and Methods. (a) Partial displacement of CBI by carbamoyl phosphate. Difference spectrum without carbamoyl phosphate (1) and with 14.8 (2), 20.6 (3), and 28.1 mM carbamoyl phosphate (4); base line (5). (b) Displacement of CBI by ATP. Difference spectrum without ATP (1) and with 6.7 (2), 18.9 (3), 38 (4), and 48.7 mM ATP (5); base line (6). (c) Effect of CTP on the difference spectrum. Base line (1); difference spectrum without CTP (2) and with 16 (3), 29.6 (4), and 41.3 mM CTP (5).

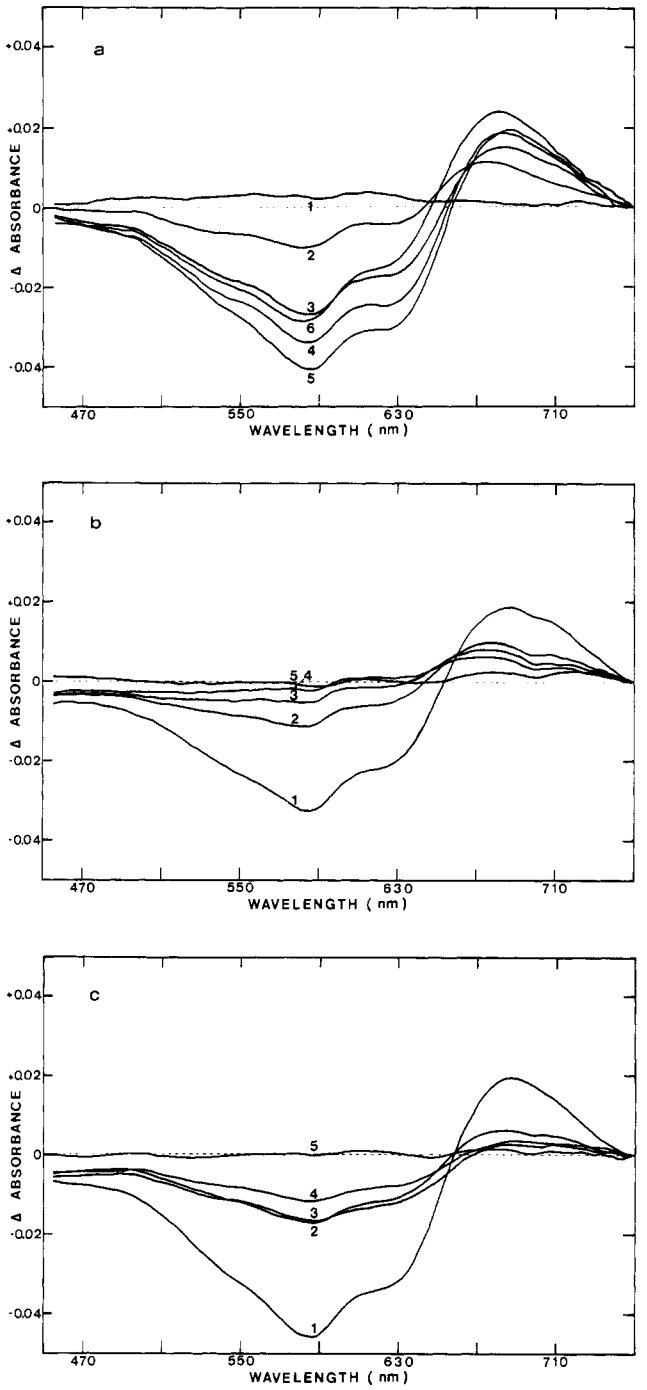

FIGURE 9: Difference spectra of CBI in the presence of isolated regulatory subunits in the absence and presence of ATP and CTP. The difference spectra were determined (a) without Cibacron Blue (1) (base line) and with 9.9 (2), 19.6 (3), 29.1 (4), 47.6 (5), and 141.5 μM CBI (6), in the presence of 10.6 μM regulatory subunits under the conditions described under Materials and Methods. The buffer was 10 mM imidazole-HCl (pH 7) containing 10 mM 2-mercaptoethanol. (b) Displacement of 0.16 mM CBI by ATP. Difference spectrum without ATP (1) and with 8.6 (2), 16.6 (3), and 20 mM ATP (4); base line (5). (c) Displacement of 0.16 mM CBI by CTP. Difference spectrum without CTP (1) and with 3.50 (2), 8.7 (3), and 24 mM CTP (4); base line (5).

Cibacron Blues bind to ATCase on the same sites as the different physiological ligands of this enzyme. Most probably, this binding is restricted to these sites, as shown by the ligand-mediated abolition of the difference spectra exhibited by the dyes in the presence of catalytic and regulatory subunits. Thus, Cibacron Blues promise to be a useful probing tool for further investigations of these sites.

Acknowledgments

We are indebted to G. Aranda and B. Poiret (Ecole Polytechnique, Palaiseau) for the preliminary analysis of both dyes by infrared spectroscopy, to E. Bricas (Université d'Orsay) for permitting us the utilization of the Cary Model 118 spectrophotometer, to Drs. D. Pantaloni (Laboratoire d'En-

zymologie du C.N.R.S., Gif-sur-Yvette) and C. Ghelis (Université d'Orsay) for stimulating discussions, and to Dr. D. Shire for reading and improving the manuscript.

References

Allewell, N. M., Friedland, J., & Niekamp, K. (1975) *Biochemistry 14*, 224–230.

Apps, D. K., & Gleed, C. D. (1976) *Biochem. J. 159*, 441–443.

Ashton, A. R., & Polya, G. M. (1978) *Biochem. J. 175*, 501–506.

Baron, M. H., Lipscomb, W. N., & Evans, D. R. (1979) *J. Biol. Chem. 254*, 10686–10693.

Beck, C. F., & Howlett, G. J. (1977) *J. Mol. Biol. 111*, 1–17.

Beissner, R. S., & Rudolph, F. B. (1978) *Arch. Biochem. Biophys. 189*, 76–80.

Beissner, R. S., Quiocho, F. A., & Rudolph, F. B. (1979) *J. Mol. Biol. 134*, 847–850.

Bethell, M. R., & Jones, M. E. (1964) *Arch. Biochem. Biophys. 104*, 438–447.

Biellmann, J. F., Samama, J. P., Bränden, C. I., & Eklund, H. (1979) *Eur. J. Biochem. 102*, 107–110.

Böhme, H. J., Kopperschlägen, G., Schulz, J., & Hofmann, E. (1972) *J. Chromatogr. 69*, 209–214

Brabson, J. S., & Switzer, R. L. (1975) *J. Biol. Chem. 250*, 8664–8669.

Buckman, T. (1970) *Biochemistry 9*, 3255–3265.

Chambers, B. B., & Dunlap, R. B. (1979) *J. Biol. Chem. 254*, 6515–6521.

Changeux, J. P., Gerhart, J. C., & Schachman, H. K. (1968) *Biochemistry 7*, 531–538.

Collins, K. D., & Stark, G. R. (1969) *J. Biol. Chem. 244*, 1869–1877.

Collins, K. D., & Stark, G. R. (1971) *J. Biol. Chem. 246*, 6599–6605.

Cook, R. A. (1972) *Biochemistry 11*, 3792–3797.

Dixon, M. (1953) *Biochem. J. 55*, 170–171.

Dixon, M. (1964) in *Enzymes*, 2nd ed., pp 315–359, Longmans, Green and Co., Ltd., London.

Edwards, R. A., & Woody, R. W. (1977) *Biochem. Biophys. Res. Commun. 79*, 470–476.

Edwards, R. A., & Woody, R. W. (1979) *Biochemistry 18*, 5197–5204.

Gerhart, J. C. (1970) *Curr. Top. Cell. Regul. 2*, 275–325.

Gerhart, J. C., & Pardee, A. B. (1962) *J. Biol. Chem. 237*, 891–896.

Gerhart, J. C., & Pardee, A. B. (1964) *Fed. Proc., Fed. Am. Soc. Exp. Biol. 23*, 727–735.

Gerhart, J. C., & Holoubek, H. (1967) *J. Biol. Chem. 242*, 2886–2892.

Glazer, A. N. (1970) *Proc. Natl. Acad. Sci. U.S.A. 65*, 1057–1063.

Grazi, E., DiIasio, A., Trombetta, G., & Magri, E. (1978) *Arch. Biochem. Biophys. 190*, 405–408.

Greenwell, P. E., Jewett, S. L., & Stark, G. R. (1973) *J. Biol. Chem. 248*, 5994–6001.

Hammes, G. G., Porter, R. W., & Wu, C. W. (1970) *Biochemistry 9*, 2992–2994.

Hammes, G. G., Porter, R. W., & Stark, G. R. (1971) *Biochemistry 10*, 1046–1050.

Heyde, E. (1973) *Biochim. Biophys. Acta 293*, 351–358.

Honzatko, R. B., Monaco, H. L., & Lipscomb, W. N. (1979) *Proc. Natl. Acad. Sci. U.S.A. 76*, 5105–5109.

Jacobsberg, L. B., Kantrowitz, E. R., & Lipscomb, W. N. (1975) *J. Biol. Chem. 250*, 9238–9240.

Jacobson, G. R., & Stark, G. R. (1973a) *Enzymes, 3rd Ed. 9*, 225–308.

Jacobson, G. R., & Stark, G. R. (1973b) *J. Biol. Chem. 248*, 8003–8014.

Kantrowitz, E. R., Jacobsberg, L. B., Landfear, S. M., & Lipscomb, W. N. (1977) *Proc. Natl. Acad. Sci. U.S.A. 74*, 111–114.

Kantrowitz, E. R., Pastra-Landis, S. C., & Lipscomb, W. N. (1980a) *Trends Biochem. Sci. (Pers. Ed.) 5*, 124–128.

Kantrowitz, E. R., Pastra-Landis, S. C., & Lipscomb, W. N. (1980b) *Trends Biochem. Sci. (Pers. Ed.) 6*, 150–153.

Kerbiriou, D., & Hervé, G. (1972) *J. Mol. Biol. 64*, 379–392.

Kerbiriou, D., & Hervé, G. (1973) *J. Mol. Biol. 78*, 687–702.

Kerbiriou, D., Hervé, G., & Griffin, J. H. (1977) *J. Biol. Chem. 252*, 2881–2890.

Kleppe, K. (1966) *Biochim. Biophys. Acta 122*, 450–461.

Kumar, S. A., & Krakow, J. S. (1977) *J. Biol. Chem. 252*, 5724–2728.

Land, M., & Byfield, P. G. H. (1979) *Int. J. Biol. Macromol. 1*, 223–226.

Lauritzen, A. M., Landfear, S. M., & Lipscomb, W. N. (1980) *J. Biol. Chem. 255*, 602–607.

London, R. E., & Schmidt, P. G. (1972) *Biochemistry 11*, 3136–3142.

London, R. E., & Schmidt, P. G. (1974) *Biochemistry 13*, 1170–1179.

Lowry, O. H., Rosebrough, N. J., Farr, A. L., & Randall, R. J. (1951) *J. Biol. Chem. 193*, 265–275.

Matsumoto, S., & Hammes, G. G. (1973) *Biochemistry 12*, 1388–1394.

Moe, J. G., & Piszkiewicz, D. (1979) *Biochemistry 18*, 2810–2814.

Monaco, H. L., Crawford, J. L., & Lipscomb, W. N. (1978) *Proc. Natl. Acad. Sci. U.S.A. 75*, 5276–5280.

Nissler, K., Kessler, R., Schellenberger, W., & Hofmann, E. (1979) *Biochem. Biophys. Res. Commun. 91*, 1462–1467.

Perbal, B., & Hervé G. (1972) *J. Mol. Biol. 70*, 511–529.

Pompon, D., & Lederer, F. (1978) *Eur. J. Biochem. 90*, 563–569.

Pompon, D., Guiard, B., & Lederer, F. (1980) *Eur. J. Biochem. 110*, 565–570.

Porter, R. W., Modebe, M. O., & Stark, G. R. (1969) *J. Biol. Chem. 244*, 1846–1859.

Ramesh, K. S., & Rao, N. A. (1980) *Biochem. J. 187*, 249–252.

Schaffer, M. H., & Stark, G. R. (1972) *Biochem. Biophys. Res. Commun. 46*, 2082–2085.

Schuber, F., & Pascal, M. (1977) *Biochimie 59*, 735–737.

Stellwagen, E. (1977) *Acc. Chem. Res. 10*, 92–98.

Suter, P., & Rosenbusch, J. P. (1976) *Eur. J. Biochem. 70*, 191–196.

Suter, P., & Rosenbusch, J. P. (1977) *J. Biol. Chem. 252*, 8136–8141.

Thiry, L., & Hervé, G. (1978) *J. Mol. Biol. 125*, 515–534.

Thompson, S. T., & Stellwagen, E. (1976) *Proc. Natl. Acad. Sci. U.S.A. 73*, 361–365.

Thompson, S. T., Cass, K. H., & Stellwagen, E. (1975) *Proc. Natl. Acad. Sci. U.S.A. 72*, 669–672.

Tondre, C., & Hammes, G. G. (1974) *Biochemistry 13*, 3131–3136.

Toste, A. P., & Cooke, R. (1979) *Anal. Biochem. 95*, 317–328.
Van Derliyn, P., Barrio, J. R., & Leonard, N. J. (1978) *J. Biol. Chem. 253*, 8694–8696.
Webb, J. L. (1963) in *Enzyme and Metabolic Inhibitors*, Vol. 1, pp 507–512, Academic Press, New York.
Weber, B. H., Willeford, K., Moe, J. C., & Piszkiewicz, D. (1979) *Biochem. Biophys. Res. Commun. 86*, 252–258.
Wedler, F. C., & Gasser, F. J. (1974) *Arch. Biochem. Biophys. 163*, 57–78.
Wild, J. R., Foltermann, K. F., & O'Donovan, G. A. (1980) *Arch. Biochem. Biophys. 201*, 506–517.
Wilson, J. E. (1976) *Biochem. Biophys. Res. Commun. 72*, 816–824.
Winlund, C. C., & Chamberlin, M. J. (1970) *Biochem. Biophys. Res. Commun. 40*, 43–49.
Winlund-Gray, C., Chamberlin, M. J., & Gray, D. M. (1973) *J. Biol. Chem. 248*, 6071–6079.
Witt, J. J., & Roskoski, R., Jr. (1980) *Biochemistry 19*, 143–148.
Yon, R. (1972) *Biochem. J. 129*, 311–320.

Salt-Induced Conformational Changes in the Catalytic Subunit of Adenosine Cyclic 3′,5′-Phosphate Dependent Protein Kinase. Use for Establishing a Connection between One Sulfhydryl Group and the γ-P Subsite in the ATP Site of This Subunit†

Juan S. Jiménez,‡ Abraham Kupfer,§ Viviane Gani,‖ and Shmuel Shaltiel*,⊥

ABSTRACT: The sulfhydryl groups in the catalytic subunit (C) of cAMP-dependent protein kinase are shown to behave as built-in reporter groups, whose relative chemical reactivity can be used to demonstrate that C readily undergoes a salt-induced conformational change at neutral pH and around physiological values of ionic strength. Upon increasing the ionic strength of the medium from 0.03 to 0.22, one SH group in C becomes more reactive toward 5,5′-dithiobis(2-nitrobenzoic acid) (the rate constant increases ~4.5-fold) while the other SH group in C becomes less reactive toward the same reagent (the rate constant decreases ~3.8-fold). Modification of the SH groups of C by this reagent brings about an inactivation of the enzyme which, at low ionic strength, can be shown to occur concomitantly and stoichiometrically with the modification of one (kinetically characterized) sulfhydryl. When ATP and its analogues are used to protect the enzyme from inactivation by this reagent, a connection is established between this SH group and the γ-P subsite of the ATP binding site in C. In parallel with the above-mentioned salt-induced conformational change, the C subunit undergoes an inactivation (which increases with ionic strength) as measured by histone H2b phosphorylation. Though not reflected in the V_{max}, this conformational change considerably increases the K_m of the enzyme for histone H2b (~4-fold) as well as for MgATP (~3.4-fold). This intrinsic malleability of the enzyme, shown here to occur even in the absence of substrate, can account for the well-known salt inhibition of the enzyme for certain substrates and the ion-dependent activation toward other substrates. It can also account for the somewhat contradictory results reported from different laboratories with regard to the functional role of the sulfhydryl groups in the enzyme. It is suggested that this intrinsic malleability might constitute a molecular basis for modulating the specificity of the enzyme and targeting its activity from one substrate to another in response to intracellular specifier signals.

Chemical modification of the sulfhydryl groups in the catalytic subunit (C)[1] of cAMP-dependent protein kinase was studied in several laboratories (Sugden et al., 1976; Kochetkov, et al., 1976; Bechtel et al., 1977; Peters et al., 1977; Armstrong & Kaiser, 1978; Kupfer et al., 1979). However, the conclusions reached regarding the functional assignment(s) of these groups were significantly different; Sugden et al. (1976) found that the bovine liver enzyme contains one SH group per C subunit and that this sulfhydryl is essential for activity. Kochetkov et al. (1976) showed that the C subunit of the porcine brain enzyme contains three cysteine residues which can be blocked without a substantial effect on the catalytic activity of the enzyme. Bechtel et al. (1977) reported that the binding of 1 mol of *N*-ethylmaleimide per mol of C from rabbit skeletal muscle results in almost complete (>85%) inactivation of the enzyme. Peters et al. (1977) carried out a stoichiometric titration of the sulfhydryl groups in bovine heart C with Nbs_2 and came to the conclusion that inactivation of the enzyme by reaction with this reagent results from the modification of two SH groups. Furthermore, they provided evidence suggesting that none of the SH groups in the enzyme is directly involved in catalysis, since a percyanylated derivative of the enzyme retained 63% of the activity of the enzyme. Working

† From the Department of Chemical Immunology, The Weizmann Institute of Science, Rehovot, Israel. *Received August 5, 1980.* A preliminary report on part of the results reported here was published (Kupfer et al., 1980).

‡ Permanent address: Department of Physical Chemistry, University of Granada, Granada, Spain. Recipient of an EMBO postdoctoral fellowship.
§ Part of a Ph.D. thesis submitted to The Feinberg Graduate School of The Weizmann Institute of Science.
‖ Permanent address: CNRS, Thiais, France.
⊥ Incumbent of the Hella and Derrick Kleeman Chair in Biochemistry.

[1] Abbreviations: AMP-PNP, adenosine 5′-(β,γ-imidotriphosphate); C, the catalytic subunit of cAMP-dependent protein kinase; cAMP, adenosine cyclic 3′,5′-phosphate; Nbs_2, 5,5′-dithiobis(2-nitrobenzoic acid); EDTA, ethylenediaminetetraacetic acid; Hepes, *N*-(2-hydroxyethyl)-piperazine-*N*′-2-ethanesulfonic acid.

0006-2960/82/0421-1623$01.25/0