Reactivity of the β Subunit of *Escherichia coli* Adenosine Triphosphatase with 4-Chloro-7-nitrobenzofurazan†

Joël Lunardi, Michel Satre, Mireille Bof, and Pierre V. Vignais*

ABSTRACT: Interaction of 4-chloro-7-nitrobenzofurazan (Nbf) with soluble bacterial ATPase (BF_1-ATPase) from *Escherichia coli* was investigated by kinetic and binding studies. As shown by kinetic studies, reaction of 1 mol of Nbf per active site of BF_1-ATPase resulted in full inactivation of the enzyme. Complete inactivation was accompanied by covalent binding of 1 mol of [^{14}C]Nbf per mol of enzyme. Nbf bound specifically to subunit β in BF_1-ATPase. At neutral pH, it reacted predominantly with a tyrosyl residue of this subunit, and the bound Nbf could be efficiently displaced by dithiothreitol. Upon alkalinization, the bound Nbf was slowly transferred to an amino group in the same subunit. Nbf was also able to bind to isolated β subunit at neutral pH, and the binding stoichiometry at saturation was 1 mol of Nbf per mol of subunit; in this case, the bound Nbf was hardly displaced by dithiothreitol. The interaction of Nbf with ADP/ATP sites was studied by following the fluorescence of the aurovertin–BF_1-ATPase complex. Nbf did not modify the enhancement of fluorescence induced by ADP, but it inhibited the quenching of fluorescence caused by ATP. The effect of Nbf on the photolabeling of BF_1-ATPase by arylazido-ADP or -ATP was also examined. Upon photoirradiation in the absence of Nbf, arylazido-ADP or arylazido-ATP bound covalently to the α and β subunits. Preincubation of BF_1-ATPase with Nbf prior to photoirradiation prevented the binding of these photoactivable derivatives to subunit β but not to subunit α. It was concluded that the Nbf binding site in BF_1-ATPase is on subunit β, close to or identical with the ADP/ATP site, and that ATP hydrolysis by BF_1-ATPase is compatible with a mechanism of half-site reactivity.

The adenine analogue 4-chloro-7-nitrobenzofurazan (Nbf)[1] has been used extensively to investigate a reactive tyrosyl group at the active site of H^+-linked ATPases. Binding of Nbf to this tyrosyl group inhibits the hydrolytic activity of mitochondrial F_1-ATPase (Ferguson et al., 1975a). After displacement of the bound Nbf by dithiothreitol, the enzymatic activity is restored. At alkaline pH, the bound Nbf undergoes an intramolecular shift from the tyrosyl group to an amino group to give a stable N-Nbf derivative, and the resulting ATPase becomes irreversibly inhibited (Ferguson et al., 1975b). Not only does Nbf inhibit mitochondrial F_1-ATPase but also it inhibits chloroplastic CF_1-ATPase (Deters et al., 1975; Cantley & Hammes, 1975) and bacterial BF_1-ATPase (Futai et al., 1974; Nelson et al., 1974; Verheijen et al., 1978).

Whereas there is general agreement regarding the binding and inhibitory properties of Nbf when interacting with F_1-ATPase, there remain controversies as to the binding stoichiometry of Nbf with BF_1-ATPase and as to which subunit of BF_1-ATPase binds Nbf. For example, Nelson et al. (1974) reported that Nbf binds to the β subunit whereas Verheijen et al. (1978) found that Nbf binds predominantly to the α subunit. The stoichiometry of BF_1 inactivation is also argued. Nelson et al. (1974) claimed that full inhibition requires 2–3 mol of Nbf per mol of enzyme, and, in agreement with this result, Futai et al. (1974) reported that BF_1-ATPase loses 50% of its activity upon binding 1 mol of Nbf per mol of enzyme. On the other hand, Verheijen et al. (1978) found that binding of 0.8 mol of Nbf to the α subunit and 0.4 mol of Nbf to the β subunit per mol of BF_1-ATPase results in 40% inhibition. Although they suggest that binding of 1 mol of Nbf to 1 β subunit might be essential to cause full inhibition, it could be inferred from their results that full inhibition requires 2 mol of Nbf bound per mol of enzyme. Since binding curves were not presented in these papers, it is not possible to differentiate high- and low-affinity sites for Nbf. Finally, there is no data on the characterization of the amino acid residue(s) of BF_1-ATPase which react(s) with Nbf. Because the effect of Nbf on BF_1-ATPase was only briefly mentioned in the above reports, and the reported data were contradictory, we thought that an extended investigation was suitable to determine accurately the binding and inhibitory properties of Nbf with respect to BF_1-ATPase. This investigation was also suitable for determining the extent to which the binding and inhibitory properties of Nbf with respect to BF_1-ATPase are similar to those reported for F_1-ATPase.

The results presented here show that Nbf binding occurs predominantly at the level of subunit β and that binding of 1 mol of Nbf per mol of BF_1-ATPase results in full inactivation of the enzyme. Because BF_1-ATPase possesses at least two copies of subunit β, this inactivation stoichiometry strongly suggests that BF_1-ATPase displays half of the site reactivity with Nbf. These results are similar to those reported by Ferguson et al. (1974) for F_1-ATPase and by Cantley & Hammes (1975) and Deters et al. (1975) for CF_1-ATPase. Furthermore, observations of the interaction of Nbf with ATP binding indicates that the Nbf site is closely related to the catalytic site.

Materials and Methods

Materials. [^{14}C]Nbf (109 mCi/mmol) was obtained from the Commissariat à l'Energie Atomique (Centre d'Etudes

† From Laboratoire de Biochimie, Département de Recherche Fondamentale, Centre d'Etudes Nucléaires, 85X, 38041 Grenoble cedex, et Faculté de Médecine de Grenoble, France. *Received June 21, 1979.* This investigation was supported by research grants from the Centre National de la Recherche Scientifique (E.R.A. No. 36), the Fondation pour la Recherche Médicale, and the Délégation Générale à la Recherche Scientifique et Technique.

[1] Abbreviations used: Nbf, 4-chloro-7-nitro-2,1,3-oxadiazole or 4-chloro-7-nitrobenzofurazan; DCCD, dicyclohexylcarbodiimide; Mops, 3-(*N*-morpholino)propanesulfonic acid; [^{3}H]arylazido-ADP, 3′-*O*-[4-[*N*-(4-azido-2-nitrophenyl)amino]butyryl]ADP; [^{3}H]arylazido-ATP, 3′-*O*-[4-[*N*-(4-azido-2-nitrophenyl)amino]butyryl]ATP; BF_1-ATPase, soluble bacterial ATPase; F_1-ATPase, soluble mitochondrial ATPase; CF_1-ATPase, soluble chloroplastic ATPase; DTT, dithiothreitol; $NaDodSO_4$, sodium dodecyl sulfate.

0006-2960/79/0418-5310$01.00/0

Nucléaires, Saclay, France). [^{14}C]Nbf was found to be more than 97% pure by thin-layer chromatography on silica gel (Merck 60 F 254) developed with cyclohexane–acetone (70:30 v/v). Unlabeled Nbf and [^{14}C]Nbf were used as ethanolic solutions, stored at −20 °C, and protected from light. Appropriate controls were run with ethanol alone, the final ethanol concentration being always less than 2%.

Aurovertin D, purified from cultures of *Calcarisporium arbuscula* (NRRL 3705) (Osselton et al., 1974), was also stored in the dark at −20 °C as an ethanolic solution. The molar extinction coefficient of our aurovertin D preparation was 35 100 M^{-1} cm^{-1} at 368 nm. Aurovertin fluorescence was measured at 30 °C with a Perkin-Elmer MPF-2A fluorometer. The excitation wavelength was set at 365 nm, and the emission wavelength was set at 470 nm.

[^{3}H]Arylazido-ADP [3′-*O*-[4-[*N*-(4-azido-2-nitrophenyl)-amino]butyryl]ADP] and [^{3}H]arylazido-ATP [3′-*O*-[4-[*N*-(4-azido-2-nitrophenyl)amino]butyryl]ATP] were synthesized by the method of Jeng & Guillory (1975), starting from [4-^{3}H]aminobutyric acid (45 Ci/mmol) (Lunardi et al., 1977) obtained from the Commissariat à l'Energie Atomique (Centre d'Etudes Nucléaires, Saclay, France). Their final specific radioactivity was 280 dpm/pmol.

The *Escherichia coli* strain used in this study was AN180 (arg E3, thi-1) (Butlin et al., 1971). Bacteria were grown at 37 °C on peptone–yeast extract medium (Miller, 1972), harvested in the late logarithmic phase, and stored at −80 °C.

BF_1-ATPase Purification. Membranes were prepared by disruption of the *E. coli* cells in a Sorvall-Ribi press (Roisin & Kepes, 1972). BF_1-ATPase was released from the membranes by chloroform treatment (Beechey et al., 1975) and purified by ion-exchange chromatography on a DEAE-cellulose column (Whatman DE-52) at 4 °C using a 50–750 mM Tris-HCl gradient containing 2.5 mM 2-mercaptoethanol, 2 mM EDTA, 1 mM ATP, pH 7.4, and 20% methanol (v/v) (Vogel & Steinhart, 1976). Active fractions, eluted at approximately 0.2 M Tris-HCl, were pooled and concentrated by precipitation of the enzyme with poly(ethylene glycol) 6000. ATPase was further purified by gel filtration on a Sepharose 6B column equilibrated with the starting buffer. Purified BF_1-ATPase was stored at 0–4 °C at about 10 mg/mL. Its specific activity varied between 25 and 35 μmol/(min mg of protein) under the assay conditions described below. Binding constants were calculated by using a molecular weight of 320 000 (Satre & Zaccaï, 1979).

Isolation of BF_1-ATPase Subunits. BF_1-ATPase was dissociated by freezing in salt solution, and subunits were separated on DEAE-cellulose as described by Vogel & Steinhart (1976), except that subunits were eluted with a 0–1 M LiCl linear gradient in 50 mM Mops, 5 mM ATP, and 2.5 mM 2-mercaptoethanol, pH 6.5. Binding data were calculated by using a molecular weight of 56 000 for the α subunit and 52 000 for the β subunit (Vogel & Steinhart, 1976).

ATPase Assay. ATPase activity was assayed at 30 °C with an ATP-regenerating system. ATP hydrolysis was initiated by addition of an aliquot fraction of BF_1 (3–5 μg) in a medium containing 4 mM phosphoenolpyruvate, 20 μg of pyruvate kinase, 10 mM ATP, 5 mM $MgCl_2$, and 40 mM Tris-HCl, pH 8.0, in a final volume of 0.5 mL. After incubation for 5 min, the reaction was terminated by addition of 0.2 mL of trichloroacetic acid (50% w/v). The phosphate released by ATP hydrolysis was measured by the procedure of Fiske & SubbaRow (1925).

Protein. Protein concentration was measured according to Bradford (1976) with bovine serum albumin as the standard.

Inactivation and Labeling with Nbf. Inactivation of soluble and membrane-bound BF_1-ATPase by Nbf was assayed in 200 mM sucrose, 2 mM EDTA, and 10 mM triethanolamine buffer, pH 7.5, at 30 °C and in the dark to prevent photolytic decomposition of Nbf (Ferguson et al., 1975a). For [^{14}C]Nbf binding, BF_1-ATPase in the above buffer was reacted with [^{14}C]Nbf and then filtered through a Sephadex G-50 (fine) column (Penefsky, 1977) to remove the unreacted [^{14}C]Nbf. The excluded fraction was assayed for ATPase activity, protein content, and ^{14}C radioactivity. At this stage, the excluded radioactive fraction contained the [^{14}C]Nbf bound to tyrosine and to any reactive thiols or amino residues. This fraction was then incubated with 1 mM dithiothreitol for 30 min at 20 °C to displace the [^{14}C]Nbf bound to tyrosine or thiol residues and filtered as described above. Assays controls were performed by using BF_1-ATPase under the same conditions except that Nbf was omitted.

Spectral Determinations. Spectral modifications resulting from the interaction of BF_1-ATPase with Nbf were followed by using a Cary 15 spectrophotometer thermostated at 30 °C. Spectra were recorded at various times during incubation and, concomitantly, aliquots of BF_1-ATPase were removed to follow ATPase activity. Other reagents or conditions were as specified in the text.

Polyacrylamide Gel Electrophoresis. Sodium dodecyl sulfate–polyacrylamide gel electrophoresis was performed as described by Weber & Osborn (1969) using 10% acrylamide gels. As Nbf bound at neutral pH is displaced by thiols, the ATPase sample was not treated with 2-mercaptoethanol, as is usual for a number of proteins. The [^{14}C]Nbf-labeled BF_1-ATPase was preincubated with 2% $NaDodSO_4$ containing 1 mM unlabeled Nbf, prior to electrophoresis (Verheijen et al., 1978), to avoid any intramolecular transfer of the bound [^{14}C]Nbf within the denatured enzyme. Electrophoresis was carried out for 16 h using 5 mA/gel. After staining and destaining, we scanned and then sliced the gels. Each slice (1 mm) was digested overnight with 1 mL of 15% H_2O_2 at 60 °C, and radioactivity was measured by liquid scintillation counting. Recovery of the ^{14}C radioactivity was typically 30–35%, most of the losses having occurred during staining and destaining processes.

Photolabeling Assays. In photolabeling assays, BF_1-ATPase was incubated for 15 min in the dark in 0.2 M sucrose, 30 mM Tris–acetate, and 2 mM EDTA buffer, pH 7.5, with added arylazido-ADP or -ATP in a small glass tube; the tube was then rotated horizontally at 150 rpm under light (Lunardi et al., 1977). Following photoirradiation, the samples were incubated with 6 mM ADP or ATP to displace the noncovalently bound arylazido nucleotides. The remaining bound radioactivity was determined by the elution–centrifugation method described by Penefsky (1977). Controls for radioactivity background were performed by adding a large excess of ADP or ATP to samples before photoirradiation (6 mM).

Results

Time Course and Stoichiometry of Inactivation of BF_1-ATPase by Nbf. Soluble BF_1-ATPase was inhibited upon incubation with Nbf at pH 7.5. The time course of inactivation followed first-order kinetics over the range investigated; generally, at least 80% inactivation was attained (Figure 1). The half-time of inactivation, $t_{1/2}$, varied as a function of Nbf concentration. The number of Nbf molecules reacting per active site of BF_1-ATPase to form an inactive complex was calculated according to Levy et al. (1963) by plotting the log $t_{1/2}$ against the log Nbf concentration (Figure 1, insert). A straight line was obtained with a slope close to 1, indicating

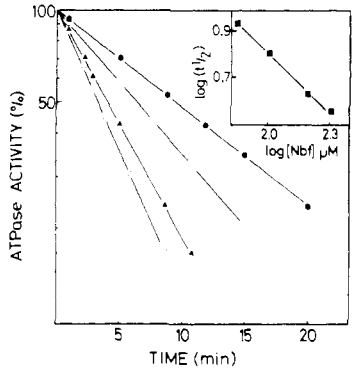

FIGURE 1: Kinetics of inactivation of soluble BF_1-ATPase by Nbf. Soluble BF_1-ATPase (0.8 mg/mL) was preincubated at 37 °C in 250 mM sucrose, 2 mM EDTA, and 10 mM triethanolamine buffer, pH 7.5, in the presence of 0.075 (●), 0.1 (○), 0.15 (▲), and 0.2 mM (△) Nbf. At various times, aliquot samples were diluted 100-fold in 0.5 mL of ATPase assay mixture and immediately assayed for ATP activity (see Materials and Methods). *Insert*: plots of the log of the half-times of inactivation ($t_{1/2}$) against the log of Nbf concentration. Values of $t_{1/2}$ (minutes) were calculated from semilogarithmic plots of ATPase activity at different Nbf concentrations.

that the kinetics is pseudo first order and also suggesting that a given molecule of BF_1-ATPase is totally inactivated upon the binding of 1 molecule of Nbf per active site.

Membrane-bound ATPase in *E. coli* vesicles was likewise inactivated by Nbf. However, the $t_{1/2}$ for inactivation of bound BF_1-ATPase was twice as much as that found for soluble BF_1-ATPase. Furthermore, in the case of membrane-bound BF_1-ATPase, the plot of log $t_{1/2}$ against log [Nbf] deviated from linearity. Only for high concentrations of Nbf was the slope close to 1. This would be explained by a delayed access of Nbf to the active site of the bound BF_1-ATPase or by nonspecific binding of Nbf to other membrane components such as proteins and phospholipids. Experiments to be described below were carried out only with soluble BF_1-ATPase.

Nature of the Nbf Binding Group in BF_1-ATPase. Binding Stoichiometry. As for F_1-ATPase (Ferguson et al., 1975a), inactivation of BF_1-ATPase by Nbf at pH 7.5 was accompanied by the appearance of an asymmetric absorption peak at 380–385 nm with a shoulder at 420 nm. Upon addition of dithiothreitol, BF_1-ATPase activity was restored and the 380–385-nm absorption peak disappeared (Figure 2). Since phenolic hydroxyl groups react with Nbf to give a derivative with an absorption peak at 380–385 nm (Aboderin et al., 1973; Ferguson et al., 1974), it may be concluded that Nbf reacts predominantly at pH 7.5 with the OH group of a tyrosyl residue of BF_1-ATPase, as in F_1-ATPase. In contrast to the main absorption peak at 380–385 nm, the shoulder at 420 nm increased with the time of incubation. The absorbance at 420 nm is due to the S-Nbf chromophore when Nbf reacts with SH group(s) in BF_1-ATPase (Ferguson et al., 1975a). The peak at 420 nm contributes somewhat to the absorbance at 380 nm, and appropriate correction must be carried out to calculate the amount of the tyrosyl-Nbf chromophore. Cantley et al. (1978) have shown that the absorbance at 380 nm due to tyrosine can be calculated by substracting 46% of the 420-nm absorbance from the 380-nm absorbance. Using this correction, a value of 11 600 M^{-1} cm^{-1} for the molar coefficient of the tyrosine–O-Nbf derivative (Ferguson et al., 1975a), and a value of 320 000 for the molecular weight of BF_1-ATPase (Satre & Zaccaï, 1979), we calculated that BF_1-ATPase is completely inactivated when 1 mol of Nbf is bound to 1 mol of BF_1-ATPase. This result was confirmed by a series of assays in which binding of [^{14}C]Nbf and inactivation of BF_1-ATPase activity were observed in parallel (Figure 3). Complete inactivation was obtained for 1 mol of [^{14}C]Nbf bound per mol of BF_1, in agreement with the spectrophotometric data. Most of the bound [^{14}C]Nbf could be displaced by dithiothreitol with concomitant and proportional recovery of ATPase activity. Increasing either the incubation time of BF_1-ATPase with [^{14}C]Nbf or the [^{14}C]Nbf concentration resulted in the binding of more than 1 mol of Nbf per mol of BF_1. This stoichiometric excess of bound Nbf was not readily removed by dithiothreitol.

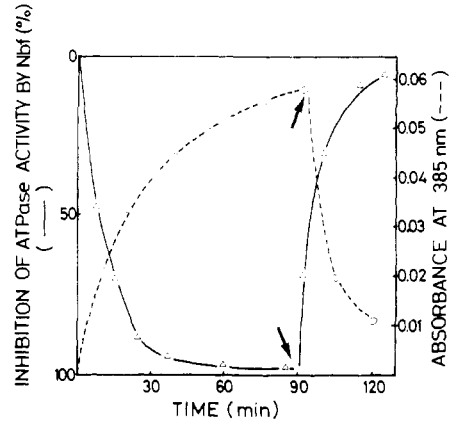

FIGURE 2: Relationship between inactivation of BF_1-ATPase by Nbf and spectral modifications. BF_1-ATPase (2.0 mg) was incubated at 30 °C in 1 mL of 250 mM sucrose, 2 mM EDTA, and 30 mM triethanolamine buffer, pH 7.5, in the presence of 50 μM Nbf. At various times, aliquots of the mixture were taken for determination of ATPase activity (as described under Materials and Methods) and optical measurements. After a 90-min incubation with Nbf, yielding 95% inactivation, the amount of bound Nbf at the tyrosyl residue (O-Nbf derivative) was calculated to be 0.93 mol/mol of enzyme (cf. Materials and Methods and Results). Dithiothreitol was then added at a final concentration of 0.5 mM (arrow).

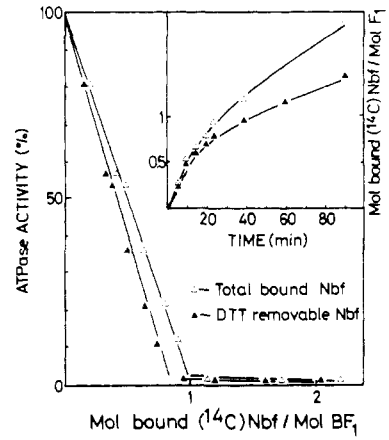

FIGURE 3: Relation between the binding of [^{14}C]Nbf to BF_1-ATPase and ATPase inactivation by bound Nbf. BF_1-ATPase (0.6 mg) was incubated in the presence of 40 μM [^{14}C]Nbf at 30 °C in 1 mL of 200 mM sucrose, 2 mM EDTA, and 20 mM triethanolamine, final pH 7.5. At time intervals and up to 90 min (cf. insert), 0.120-mL aliquots of the incubation mixture were filtered through Sephadex columns equilibrated with the same medium, except that Nbf was omitted (cf. Materials and Methods). Bound [^{14}C]Nbf and ATPase activity were assayed on small aliquots (5 μL) of the filtrates. Dithiothreitol (DTT) was then added to a final concentration of 1 mM to the remaining fractions and allowed to react for 30 min at 20 °C in the dark. The samples were again filtered on Sephadex, and the ^{14}C radioactivity was assayed in the filtrates. The two curves shown in the figure refer to the total amount of bound [^{14}C]Nbf (△) and to the amount of bound [^{14}C]Nbf removed by dithiothreitol (DTT) (▲). The insert gives the time course of incorporation of [^{14}C]Nbf (total and DTT removable in BF_1-ATPase).

Effect of Alkaline pH on the Binding of Nbf to BF_1-ATPase. Intramolecular transfer of Nbf from the OH group of a tyrosyl residue to an amino side-chain residue in beef heart

Table I: Compared Effects of Alkalinization on Reactivity of Nbf with BF_1-ATPase and F_1-ATPase[a]

enzyme	changes of pH during incubn with Nbf	[^{14}C]-Nbf incorpd[b]	A at 385 nm[c]	A at 475 nm[d]	% inactn
BF_1-ATPase	pH 7.5, 30 min	1.32	1.04	0.10	96
	pH 9.0, 30 min	1.11	0.84	0.16	84
	pH 9.0, 120 min	0.93	0.67	0.23	75
	pH 9.0, DTT, 30 min	0.37	0.05	0.28	27
F_1-ATPase	pH 7.5, 30 min	1.30	1.10	0.10	95
	pH 9.0, 30 min	1.10	0.40	0.62	90
	pH 9.0, 120 min	0.95	0.10	0.80	83
	pH 9.0, DTT, 30 min	0.90	0.02	0.84	82

[a] BF_1-ATPase (1.6 mg) and F_1-ATPase (1.5 mg) were incubated for 30 min at 30 °C in the dark in 1 mL of 200 mM sucrose, 10 mM triethanolamine, and 2 mM EDTA, pH 7.5, in the presence of 100 μM [^{14}C]Nbf. Excess of nonreacted [^{14}C]Nbf was removed by filtration of the mixture through small Sephadex G-50 columns equilibrated with the same buffer at pH 7.5. Aliquot samples were taken for measuring protein content, radioactivity, enzymatic activity, and absorbance spectrum. Then pH was brought to pH 9.0 by addition of 2 M Tris-base. The same measurements were carried out after 30 and 120 min of incubation at pH 9.0. When added, dithiothreitol (DTT) was at the final concentration of 2 mM and it was allowed to react for 30 min at 30 °C. [b] Moles of total Nbf derivatives per mole of enzyme. The total [^{14}C]Nbf incorporated consists of the sum of O-Nbf, N-Nbf, and nondetermined Nbf derivatives. [c] Moles of O-Nbf derivatives per mole of enzyme. [d] Moles of N-Nbf derivatives per mole of enzyme.

F_1-ATPase is readily obtained by raising the pH of the medium from 7.5 to 9.0 (Ferguson et al., 1975b). This O to N transfer is revealed by the appearance of a new peak at 475 nm corresponding to the formation of a lysine–N-Nbf complex that is characterized by a maximal absorbance at 475 nm and a molar absorption of 26 000 M^{-1} cm^{-1}. Spectral modifications obtained during the shift to alkaline pH for BF_1-ATPase are slightly different. After a 120-min incubation at pH 9.0 at 30 °C, absorbance at 385 nm (O-Nbf derivative) decreased by 40% but, in contrast to beef heart F_1-ATPase, the absorbance at 475 nm (N-Nbf derivative) did not increase correspondingly (see Table I). Direct assays of [^{14}C]Nbf binding also showed that, during incubation of BF_1-ATPase with Nbf at pH 9.0, the amount of bound [^{14}C]Nbf removable by dithiothreitol decreased significantly. However, the thiol-insensitive bound [^{14}C]Nbf did not increase to the same extent (see Table II). Presumably, this thiol-insensitive bound [^{14}C]Nbf corresponds to the N-Nbf-modified BF_1-ATPase; the lack of stoichiometric transfer to Nbf from the O to the N position in BF_1-ATPase may be due to loss of Nbf groups from the enzyme during the transfer process, which would explain the rise in ATPase activity observed during incubation at pH 9.0 [for example, increase from 5.1 to 10.5 μmol/(min mg) after 50 min of incubation at pH 9.0]. The lack of total recovery of activity after addition of dithiothreitol is most probably due to the accumulation of the N-Nbf derivative of BF_1-ATPase that is insensitive to dithiothreitol.

Identification of the Nbf Binding Subunit in BF_1-ATPase. To identify which subunit(s) of BF_1-ATPase react(s) with Nbf, sodium dodecyl sulfate gel electrophoresis was performed with [^{14}C]Nbf-modified enzyme (Figure 4). As indicated under Materials and Methods, electrophoresis was run in the absence of 2-mercaptoethanol. In the first series of assays, BF_1-ATPase was inactivated at pH 7.5 by [^{14}C]Nbf to roughly 90%, and

Table II: Dithiothreitol Sensitivity of the [^{14}C]Nbf Binding to BF_1-ATPase during the Alkaline pH Shift[a]

time of incubn with Nbf at pH 9.0 (min)	bound [^{14}C]Nbf (mol/mol of BF_1): removable by DTT (O-Nbf and S-Nbf derivatives)	bound [^{14}C]Nbf (mol/mol of BF_1): insensitive to DTT (N-Nbf derivative)	ATPase act. [μmol/(min mg)]: before addition of DTT	ATPase act. [μmol/(min mg)]: after reaction with DTT
0	0.90	0	5.1	29.1
15	0.81	0.01	7.2	29.0
30	0.71	0.03	9.3	27.9
50	0.62	0.08	10.5	26.1
90	0.52	0.18	11.1	24.6
140	0.43	0.22	11.7	23.4

[a] BF_1-ATPase (0.7 mg) was incubated for 30 min, at 30 °C, in the dark in 0.8 mL of 200 mM sucrose, 10 mM triethanolamine, and 2 mM EDTA, pH 7.5, in the presence of 66 μM [^{14}C]Nbf. The mixture was then filtered through Sephadex G-50 to remove the nonreacted [^{14}C]Nbf. After this step the enzyme was 95% inactivated. The pH was brought to 9.0 with Tris-base. At indicated times 100-μL aliquots of the mixture were filtered through Sephadex; the enzyme activity and the radioactivity of the filtrate were determined. Then dithiothreitol (DTT) was added to the filtrate to a final concentration of 1 mM and allowed to react for 30 min at 25 °C prior to filtration through Sephadex. ^{14}C radioactivity and enzymatic activity were measured on the effluent. A control experiment was carried out under the same conditions except that Nbf was omitted during incubation.

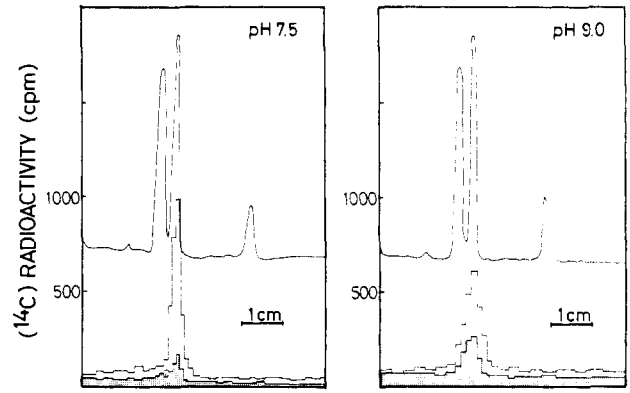

FIGURE 4: Sodium dodecyl sulfate–polyacrylamide gel electrophoresis of BF_1-ATPase labeled by [^{14}C]Nbf. BF_1-ATPase (0.3 mg) was incubated for 30 min, at 30 °C, in the dark in 0.25 mL of 200 mM sucrose, 2 mM EDTA, and 10 mM triethanolamine, pH 7.5, in the presence of 55 μM Nbf, resulting in 90% ATPase inactivation. A 0.1-mL aliquot of the mixture was then filtered through a Sephadex G-50 column equilibrated with the same buffer without Nbf at pH 7.5, and the filtrate containing the bound [^{14}C]Nbf was recovered for further $NaDodSO_4$ gel electrophoresis. Another 0.1-mL aliquot was also filtered through a Sephadex G-50 column equilibrated with a buffer containing 250 mM sucrose, 2 mM EDTA, and 20 mM Tris, pH 9.0, and the filtrate was allowed to incubate at pH 9 for 90 min at 25 °C in the dark. Prior to gel electrophoresis, the two samples of incubated BF_1-ATPase, at pH 7.5 and pH 9.0, were denaturated in 2% $NaDodSO_4$ in the presence of 0.75 mM cold Nbf. 2-Mercaptoethanol was omitted. Aliquots containing 15 μg of BF_1-ATPase were placed on the top of 10% polyacrylamide gels. The figure shows the densitometric tracing of the stained gels and the corresponding radioactivity pattern. The shaded area is the radioactivity pattern corresponding to the bound radioactivity remaining after incubation of the Nbf-modified BF_1-ATPases (pH 7.5 or 9) with 0.5 mM dithiothreitol for 30 min at 25 °C.

then the modified enzyme was analyzed by sodium dodecyl sulfate gel electrophoresis. For prevention of any rearrangement of bound [^{14}C]Nbf in the denatured subunits, an excess of unlabeled Nbf was added to the enzyme prior to the addition of sodium dodecyl sulfate [see also Ferguson et al. (1975a)

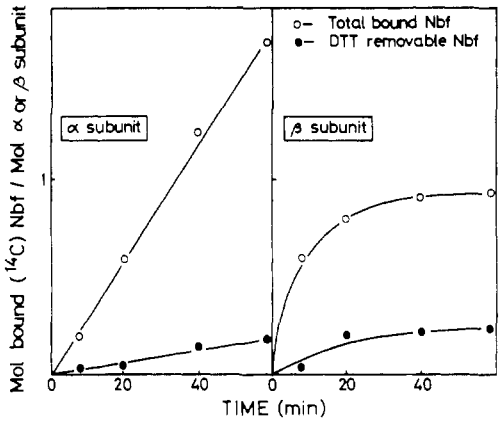

FIGURE 5: Binding curves of Nbf to isolated α and β subunits from BF_1-ATPase. The subunits were prepared as described under Materials and Methods. About 0.2 mg of protein of each subunit was allowed to react with 60 μM [^{14}C]Nbf at 30 °C in 0.5 mL of 200 mM sucrose, 10 mM Tris-HCl, and 2 mM EDTA, pH 7.5. Aliquots were removed at interval periods, and the nonreacted Nbf was eliminated by filtration on Sephadex G-50 columns (see Materials and Methods). The bound [^{14}C]Nbf recovered in the filtrates was assayed for radioactivity, and the protein content was determined.

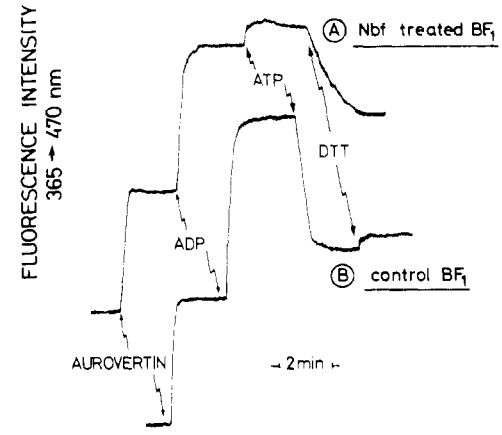

FIGURE 6: Effect of ADP, ATP, and dithiothreitol on the fluorescence intensity of the aurovertin–BF_1-ATPase complex and aurovertin–Nbf-inactivated BF_1-ATPase complex. BF_1-ATPase was inactivated to 90% by incubation with 80 μM Nbf for 30 min at 30 °C at pH 7.5. Inactivation was terminated by removal of the nonreacted Nbf by Sephadex filtration (see Materials and Methods). The fluorescence assays were made at 30 °C on control BF_1-ATPase and Nbf-modified BF_1-ATPase (200 μg of protein) incubated with 1 μM aurovertin in 2 mL of 200 mM sucrose, 20 mM Tris–acetate, and 2 mM EDTA, pH 7.5. ADP, ATP, and dithiothreitol (DTT) were added sequentially to obtain the following final concentrations: 0.1 mM ADP, 3 mM ATP, and 5 mM dithiothreitol.

and Verheijen et al. (1978)]. The radioactivity profile indicated that more than 90% of the bound radioactivity was localized on the β subunit. The bound [^{14}C]Nbf could be removed by reaction of the Nbf-modified enzyme with dithiothreitol before sodium dodecyl sulfate denaturation. Removal of the [^{14}C]Nbf is accompanied by the recovery of the ATPase activity. In a second series of assays, gel electrophoresis was performed on a sample of BF_1-ATPase first incubated with Nbf at pH 7.5 and then at pH 9.0. The bound [^{14}C]Nbf was again recovered in the β subunit, but in this case dithiothreitol was much less effective in removing the bound radioactivity. In accordance with spectrophotometric data, the nonremovable bound [^{14}C]Nbf at pH 9.0 probably corresponds to the N-Nbf derivative of BF_1-ATPase.

Binding of Nbf to Isolated α and β Subunits. Binding isotherms for the interaction of Nbf with isolated α and β subunits of BF_1-ATPase are illustrated in Figure 5. The binding curve for subunit β clearly indicated saturation for 1 mol of Nbf bound per mol of β subunit. Part of the bound Nbf was displaced by dithiothreitol. In contrast to the β subunit, the α subunit incorporated [^{14}C]Nbf linearly as a function of the time of incubation. About 1.7 mol of Nbf was found to bind per mol of α subunit after incubation with 60 μM Nbf for 1 h, without apparent tendency to saturation. The [^{14}C]Nbf bound to subunit α was scarcely displaced by dithiothreitol.

Interaction of Nbf with ADP and ATP Binding Sites. Interaction between Nbf and ADP/ATP binding sites on BF_1-ATPase was studied by two different approaches. The first was based on the fact that binding of nucleotides to BF_1-ATPase modifies the fluorescence intensity of the aurovertin–BF_1-ATPase complex; as shown in Figure 6, addition of ADP at low concentration (0.1 mM) resulted in the increase of the fluorescence of the aurovertin–ATPase complex. Subsequent addition of ATP at high concentration (3 mM) quenched the fluorescence. The complex of aurovertin and Nbf-modified BF_1-ATPase responded to the addition of ADP in the same way as the native enzyme; however, a subsequent addition of ATP did not lead to significant quenching of fluorescence. The fluorescence quenching by ATP was restored when the Nbf-modified enzyme was treated by dithiothreitol to remove Nbf.

In the second approach, we made use of two photoactivable adenine nucleotide analogues, the arylazido-ADP and -ATP. These two compounds bind covalently to the α and β subunits of BF_1-ATPase after photoactivation as described for mitochondrial F_1-ATPase (Lunardi et al., 1977). Photolabeling of nucleotide sites of BF_1-ATPase by arylazido-ADP or -ATP was shown to be specific by experiments in which more than 95% of the covalent binding of [^{3}H]arylazido nucleotides was prevented by 4 mM ADP or ATP added prior to photoirradiation. Most likely, arylazido nucleotides bound to loose binding sites of BF_1-ATPase, which rapidly exchanged their bound nucleotides with externally added ADP or ATP. Full inactivation corresponded to the binding of 2 mol of arylazido-ADP or -ATP per mol of BF_1-ATPase, which suggests that BF_1-ATPase possesses two loose binding sites for ADP or ATP. The bound arylazido-ADP or -ATP was distributed about equally between subunits α and β. When the Nbf-modified BF_1-ATPase was used instead of the native BF_1-ATPase, the extent of photolabeling was significantly decreased. In the experiment of Figure 7, Nbf was used at a concentration capable of yielding 80% inhibition of ATPase activity. [^{3}H]-Arylazido-ADP was added both to the control BF_1-ATPase and to the Nbf-modified BF_1-ATPase at a concentration of 100 μM. This concentration yielded a 70% inhibition in the native enzyme. After photoirradiation, the amount of bound [^{3}H]arylazido-ADP was 1.42 mol/mol of native enzyme and only 1.02 mol/mol of Nbf-modified enzyme. As shown by sodium dodecyl sulfate–acrylamide gel electrophoresis, the bound ^{3}H radioactivity was predominantly decreased in the β subunit of the Nbf-modified enzyme (Figure 7), indicating that the covalent binding of Nbf interferes with that of arylazido nucleotides on the β subunit of BF_1-ATPase.

Discussion

The interactions of Nbf with *E. coli* BF_1-ATPase are similar to those reported for mitochondrial F_1-ATPase (Ferguson et al., 1975a,b) and chloroplastic CF_1-ATPase (Deters et al., 1975; Cantley & Hammes, 1975). Major similarities are the selective binding of Nbf to the β subunit, probably on a tyrosine residue at neutral pH, and the occurrence of full inactivation when 1 mol of Nbf binds to 1 mol of BF_1-ATPase. However, in contrast to mitochondrial F_1-ATPase, the bound

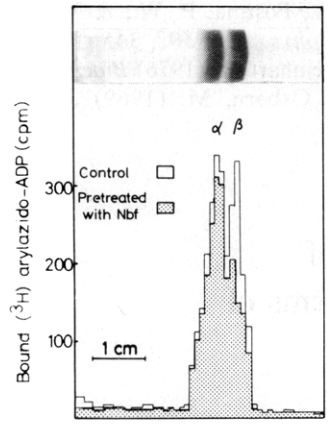

FIGURE 7: Sodium dodecyl sulfate–polyacrylamide gel electrophoresis of BF_1-ATPase photolabeled with [^{3}H]arylazido-ADP after pretreatment with Nbf. A sample (60 μg) of BF_1-ATPase was preincubated with 70 μM Nbf at 30 °C for 30 min in 0.1 mL of 200 mM sucrose, 30 mM Tris–acetate, and 2 mM EDTA, pH 7.5. This preincubation yielded an 85% inactivation. The nonreacted Nbf was eliminated by Sephadex filtration as described under Materials and Methods. The filtrate, which contained the Nbf-modified BF_1-ATPase, was recovered to be photoirradiated with 120 μM [^{3}H]arylazido-ADP at 20 °C for 30 min. A control sample of BF_1-ATPase (nontreated by Nbf) was photoirradiated with [^{3}H]arylazido-ADP, under the same conditions. Identical aliquots (8 μg) of the two enzyme samples were subjected to $NaDodSO_4$ gel electrophoresis. The figure shows the radioactivity pattern corresponding to α and β subunits of control BF_1-ATPase. The shaded columns correspond to the ^{3}H radioactivity in Nbf-modified BF_1-ATPase.

Nbf in BF_1-ATPase is transferred very slowly to an amino group upon alkalinization.

There are two lines of evidence for the interaction between Nbf and ADP/ATP on the β subunit. One is based on the binding of photoactivable derivatives of ADP or ATP. Both types of derivatives bind to BF_1-ATPase, being equally distributed in the α and β subunits. Preincubation of BF_1-ATPase with Nbf at neutral pH prior to covalent attachment of the photoactivable derivatives of ADP and ATP selectively prevents the photolabeling of the β subunit. The other line of evidence is based on the response of the aurovertin fluorescence to ATP in Nbf-modified BF_1-ATPase. In native BF_1-ATPase, ADP enhances the fluorescence intensity of the aurovertin–BF_1-ATPase complex; this enhanced fluorescence is quenched by ATP. The increase in fluorescence caused by addition of ADP to the Nbf–BF_1-ATPase–aurovertin complex is similar to that found with the native BF_1-ATPase, but no quenching is observed with ATP. The ADP and ATP sites referred to here are weak binding sites since they are able to react rapidly with added ADP or ATP; this is in contrast with the tight binding sites which are able to retain ADP or ATP in a bound form. Whereas weak sites may conceivably correspond to catalytic or regulatory sites, the strong sites have probably a regulatory function (Schuster et al., 1975).

BF_1-ATPase has to be added to a list of membrane-bound ATPases that contain a strategic tyrosine residue at the catalytic site, namely, mitochondrial F_1-ATPase (Ferguson et al., 1975a,b), chloroplastic CF_1-ATPase (Cantley & Hammes, 1975; Deters et al., 1975), and Na^+/K^+-ATPase (Cantley et al., 1978). The same as for BF_1-ATPase, F_1-ATPase and CF_1-ATPase contain at least two chains of β subunit, and the modification of only one of them by Nbf results in full inactivation. Likewise, Na^+/K^+-ATPase contains two polypeptide chains with a molecular weight of 95 000 and the modification of one of them by Nbf is necessary for complete inhibition of Na^+/K^+-ATPase activity. These data are typical of a mechanism of half-site reactivity. The half-site reactivity of BF_1-ATPase with respect to Nbf is not unique; an exactly similar situation was recently described for dicyclohexylcarbodiimide (DCCD), a carboxyl group selective reagent (Satre et al., 1979). In fact, DCCD, at appropriate pH, selectively binds to the β subunit of BF_1-ATPase, the binding of 1 mol of DCCD to 1 mol of enzyme resulting in full inhibition. The half-site reactivity of BF_1-ATPase is compatible with two catalytic mechanisms. The first one is the alternating-site mechanism proposed by Moudrianakis & Adolfsen (1975) and developed by Kayalar et al. (1977) for beef heart F_1-ATPase. According to this mechanism, the catalytic sites located on each of the β subunits of BF_1-ATPase would alternate between two states, only one of which is able to bind Nbf or DCCD; this model supposes a preexisting asymmetry of β subunits. Another possible explanation is that the binding of Nbf or DCCD to one given β subunit of BF_1-ATPase induces, by means of cooperative interactions, a conformational change in the other β subunit, which then becomes unable to bind Nbf or DCCD; this latter model, based on induction, supposes that there is no preexisting asymmetry.

References

Aboderin, A. A., Boedefeld, E., & Luisi, P. L. (1973) *Biochim. Biophys. Acta 328*, 20–30.

Beechey, R. B., Hubbard, S. A., Linnett, P. E., Mitchell, A. D., & Munn, E. A. (1975) *Biochem. J. 148*, 533–537.

Butlin, J. D., Cox, G. B., & Gibson, F. (1971) *Biochem. J. 124*, 75–81.

Cantley, L. C., Jr., & Hammes, G. G. (1975) *Biochemistry 14*, 2968–2975.

Cantley, L. C., Jr., Gelles, J., & Josephson, L. (1978) *Biochemistry 17*, 418–425.

Deters, D. W., Racker, E., Nelson, N., & Nelson, H. (1975) *J. Biol. Chem. 250*, 1041–1047.

Ferguson, S. J., Lloyd, W. J., & Radda, G. K. (1974) *FEBS Lett. 38*, 234–236.

Ferguson, S. J., Lloyd, W. J., Lyons, M. H., & Radda, G. K. (1975a) *Eur. J. Biochem. 54*, 117–126.

Ferguson, S. J., Lloyd, W. J., & Radda, G. K. (1975b) *Eur. J. Biochem. 54*, 127–133.

Fiske, C. A., & SubbaRow, Y. (1925) *J. Biol. Chem. 66*, 375–400.

Futai, M., Sternweis, P. C., & Heppel, L. A. (1974) *Proc. Natl. Acad. Sci. U.S.A. 71*, 2725–2729.

Jeng, S. J., & Guillory, R. J. (1975) *J. Supramol. Struct. 3*, 448–468.

Kayalar, C., Rosing, J., & Boyer, P. D. (1977) *J. Biol. Chem. 252*, 2486–2491.

Levy, H. M., Leber, P. D., & Ryan, E. M. (1963) *J. Biol. Chem. 238*, 3654–3659.

Lunardi, J., Lauquin, G. J. M., & Vignais, P. V. (1977) *FEBS Lett. 80*, 317–323.

Miller, J. H. (1972) in *Experiments in Molecular Genetics*, pp 201–205, Cold Spring Harbor Laboratory, Cold Spring Harbor, NY.

Moudrianakis, E. N., & Adolfsen, R. (1975) in *Electron Transfer Chains and Oxidative Phosphorylation* (Quagliariello, E., et al., Eds.) pp 373–378, North-Holland/Elsevier, Amsterdam.

Nelson, N., Kanner, B. I., & Gutnick, D. L. (1974) *Proc. Natl. Acad. Sci. U.S.A. 71*, 2720–2724.

Osselton, M. D., Baum, H., & Beechey, R. B. (1974) *Biochem. Soc. Trans. 2*, 200–202.

Penefsky, H. S. (1977) *J. Biol. Chem. 252*, 2891–2899.

Roisin, M. P., & Kepes, A. (1972) *Biochim. Biophys. Acta 275*, 333–346.

Satre, M., & Zaccai, G. (1979) *FEBS Lett. 102*, 244–248.
Satre, M., Lunardi, J., Pougeois, R., & Vignais, P. V. (1979) *Biochemistry 18*, 3134–3140.
Schuster, S. M., Ebel, R. E., & Lardy, H. A. (1975) *J. Biol. Chem. 250*, 7848–7853.
Verheijen, J. H., Postma, P. W., & Van Dam, K. (1978) *Biochim. Biophys. Acta 502*, 345–353.
Vogel, G., & Steinhart, R. (1976) *Biochemistry 15*, 208–216.
Weber, K., & Osborn, M. (1969) *J. Biol. Chem. 244*, 4406–4412.

Evaluation of Equilibrium Constants for the Binding of *N*-Acetyl-L-tryptophan to Monomeric and Dimeric Forms of α-Chymotrypsin†

Ross Tellam, John de Jersey, and Donald J. Winzor*

ABSTRACT: The binding of *N*-acetyl-L-tryptophan to the monomeric and dimeric forms of α-chymotrypsin in $I = 0.2$ acetate–chloride buffer, pH 3.86, has been studied quantitatively. Equilibrium sedimentation studies in the absence of inhibitor yielded a dimerization constant of 3.5 L/g. This value was confirmed by frontal gel chromatography of the enzyme on Bio-Gel P-30, which was also used to establish that *N*-acetyl-L-tryptophan binds preferentially to monomeric enzyme. From kinetic studies of competitive inhibition with *N*-acetyl-L-tryptophan ethyl ester as substrate, an equilibrium constant of 1300 M^{-1} was determined for the binding of *N*-acetyl-L-tryptophan to monomeric α-chymotrypsin. An intrinsic binding constant of 250 M^{-1} for the corresponding interaction with dimeric enzyme was calculated on the basis of these results and binding data obtained with concentrated (18.5 g/L) α-chymotrypsin. The present results refute earlier claims for exclusive binding of competitive inhibitors to monomer and also those for equivalence of inhibitor binding to monomeric and dimeric forms of α-chymotrypsin.

The effect of covalent active-site-directed inhibitors on the macromolecular state of α-chymotrypsin has received considerable attention [e.g., Neet & Brydon (1970), Horbett & Teller (1973), and Gorbunoff et al. (1978)] because of the close proximity or, indeed, identity of the active site and the dimerization site (Steitz et al., 1969; Aune & Timasheff, 1971; Birktoft & Blow, 1972; Vandlen & Tulinski, 1973). These studies are in general agreement that (1) inhibitor binding leads to an increase in the proportion of monomeric enzyme and (2) the extent of the shift toward monomer depends at least in part on the bulkiness of the inhibitor group introduced into the enzyme molecule.

However, no such concord extends to studies of the relative affinities of monomeric and dimeric α-chymotrypsin species for competitive (noncovalent) inhibitors. Whereas Sarfare et al. (1966) concluded, on the basis of molecular weight studies, that the binding of β-phenylpropionate proceeds independently of enzyme polymerization, Shiao & Sturtevant (1969) considered that their studies of inhibitor binding by flow microcalorimetry were best described by a model in which dimeric enzyme possessed little, if any, affinity for indole, *N*-acetyltryptophan, or proflavin. A similar conclusion was reached by Faller & LaFond (1971), who studied the binding of proflavin to α-chymotrypsin by equilibrium dialysis and temperature-jump relaxation methods. An intermediate stand was taken by Nichol et al. (1972), who combined binding data and molecular weight measurements to show that phenylpropiolate and β-phenylpropionate bind to dimeric enzyme, but with decreased affinity. Subsequently, Gilleland & Bender (1976) have considered kinetic studies of proflavin binding in terms of exclusive binding to monomeric α-chymotrypsin, whereas the original Sarfare et al. model with equivalent binding to monomeric and dimeric enzyme has returned to favor as the result of NMR studies of the binding of *p*-fluorocinnamate (Gerig et al., 1977).

Because of the uncertainty created by these conflicting viewpoints, we have investigated the binding of *N*-acetyl-L-tryptophan to α-chymotrypsin in $I = 0.2$ acetate–chloride, pH 4, a medium in which (a) polymerization of the enzyme is restricted to a monomer–dimer equilibrium (Winzor & Scheraga, 1964; Winzor et al., 1967; Morimoto & Kegeles, 1967; Aune & Timasheff, 1971; Horbett & Teller, 1973, 1974; Gorbunoff et al., 1978) and (b) the problem of autolysis in equilibrium binding studies by frontal gel chromatography (Nichol et al., 1972) is minimal. A combination of equilibrium sedimentation, gel chromatographic, and enzyme kinetic studies has been used to evaluate the binding constants for the interactions of *N*-acetyl-L-tryptophan with monomeric and dimeric α-chymotrypsin.

Experimental Section

Materials. α-Chymotrypsin (3-times crystallized, freeze-dried, and salt-free) was obtained from Worthington Biochemical Corp., *N*-acetyl-L-tryptophan was from Sigma Chemical Co., and *N*-acetyl-L-tryptophan ethyl ester was from Vega-Fox Biochemicals. Other chemicals were of reagent grade, and glass-distilled water was used in the preparation of all buffers and solutions. Acetate–chloride buffer, pH 3.86, $I = 0.20$ (0.18 M sodium chloride and 0.02 M sodium acetate; pH adjusted with acetic acid), was used throughout this investigation.

Solutions of α-chymotrypsin were prepared by direct dissolution into the acetate–chloride buffer, and any autolysis fragments were removed by zonal gel chromatography of 2-mL aliquots on a column (2.4 × 17 cm) of Sephadex G-75 preequilibrated with the same buffer. A high protein concentration (~20 g/L) such that the α-chymotrypsin would be essentially dimeric was used to improve the separation of the enzyme from

† From the Department of Biochemistry, University of Queensland, St. Lucia, Queensland 4067, Australia. *Received June 11, 1979.* Supported in part by the Australian Research Grants Committee.

0006-2960/79/0418-5316$01.00/0