Ligand Binding Studies of the F_1 Moiety of Rat Liver ATP Synthase: Implications about the Enzyme's Structure and Mechanism†

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ABSTRACT: F_1-ATPase of rat liver was examined for its capacity to interact with both metal ions and nucleotides and for the effect of covalent ATPase inhibitors on these interactions. As isolated, rat liver F_1 contains about 2 mol of Mg^{2+}/mol of F_1, 1 mol of which can be removed or exchanged. The remaining mole of Mg^{2+} per mole of F_1 remains very tightly associated with F_1 and is recovered in the $\alpha\gamma$ fraction after cold denaturation. Rat liver F_1 also contains as isolated a nearly equivalent amount of nucleotide (~1.7 mol/mol of F_1) which is readily removed by incubation at room temperature followed by column centrifugation. The "2 Mg^{2+} enzyme" binds almost 3 mol of 5'-adenylyl imidodiphosphate (AMP-PNP)/mol of F_1 in the presence or absence of added divalent cation. When divalent cation is present as Co^{2+}, an equivalent activator to Mg^{2+} in the ATPase reaction, 1 mol of F_1 binds 3 mol of both AMP-PNP and Co^{2+}. Under these conditions, the very tight Mg^{2+} site remains loaded, the exchangeable Mg^{2+} site is replaced with AMP-PNPCo, and two additional AMP-PNPCo sites are filled. At this point, ADP can be loaded onto the enzyme as a fourth nucleotide at a site separate and distinct from the AMP-PNP sites. Significantly, rat liver F_1 contains only a single readily detectable ADP binding site in the presence or absence of divalent cation. The covalent labeling agent 7-chloro-4-nitro-2,1,3-benzoxadiazole prevents binding of all three AMP-PNP molecules and two of the three Co^{2+} molecules and reduces bound ADP by about 50%. Carboxylate labeling agents and a variety of other covalent labeling agents are without effect on Co^{2+} binding to F_1. These results are best interpreted within the framework of the asymmetric structure of the rat liver F_1 complex which contains only a single copy of the smaller subunits ($\gamma\delta\epsilon$) per three $\alpha\beta$ pairs. It is suggested that the ADP site, the tight Mg^{2+} site, and the exchangeable Mg^{2+} site (capable of forming an $\overline{E}$-Mg-ATP complex) lie at the asymmetric center of F_1, i.e., on an $\alpha\beta$ pair "tagged" by one or more of the smaller subunits. In contrast, the two additional ATP metal binding sites are considered to lie on "naked" or "pure" $\alpha\beta$ subunit pairs. Three current models for the function of F_1 are discussed with respect to their capacity to accommodate the ligand binding data obtained for the rat liver enzyme.

Proton ATPases of the F_0F_1 type are found in bacteria, chloroplasts, and mitochondria [for recent references, see Senior (1979a), Dunn & Heppel (1981), Amzel & Pedersen (1983), Senior & Wise (1983), and Wang (1983)]. These enzymes have a bifunctional capacity which is expressed to different degrees throughout the phylogenetic scale. In certain anaerobic bacteria, the F_0F_1 complex functions mainly as a "coupled" ATPase in supporting a variety of ATP-dependent processes whereas in most animal and plant cells its main function is in ATP synthesis. In some organisms, e.g., *Escherichia coli*, it may function in both directions.

The catalytic unit of the F_0F_1 class of ATPases is the F_1 moiety. F_1 preparations from various sources exhibit a molecular weight of 360K–380K and contain five nonidentical subunits in the stoichiometric ratio $\alpha_3\beta_3\gamma\delta\epsilon$ (Catterall & Pedersen, 1971; Senior & Brooks, 1971; Bragg & Hou, 1975; Kagawa et al., 1976; Foster & Fillingame, 1979). Of the three $\alpha\beta$ heterodimers characteristic of the F_1 moiety, one may be unique because of its interaction with the smaller subunits which are present in only a single copy. In fact, direct evidence for such an "asymmetric center" in F_1 has been derived recently both from cold dissociation studies (Williams et al., 1984a) and from cross-linking studies with a water-soluble carbodiimide (Lotscher & Capaldi, 1984).

Results presented below summarizing ligand binding data on rat liver F_1 provide additional support for subunit asymmetry within this enzyme complex.

Experimental Procedures

Materials

Rats were obtained from Charles River Breeding Laboratories. ATP and ITP were obtained from P-L Biochemicals whereas GTP was obtained from Boehringer Mannheim. 5'-Adenylyl imidodiphosphate (AMP-PNP),[1] divalent cation chloride complexes, EDTA, Hepes, Tris-HCl, glycylglycine, luciferin, luciferase, phenylglyoxal, 2,3-butanedione, Woodward's reagent K, and EDAC were purchased from Sigma Chemical Co. Nitric acid, potassium phosphate, and ammonium sulfate were from J. T. Baker Chemical Co., Chelex-100 was from Bio-Rad, and Sephadex G-25 was from Pharmacia. DCCD was obtained from Schwarz/Mann and NBD-Cl from Nutritional Biochemical Corp. Tuberculin

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[1] Abbreviations: AMP-PNP, 5'-adenylyl imidodiphosphate; Hepes, 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid; DCCD, dicyclohexylcarbodiimide; EDAC, 1-ethyl-3-[3-(dimethylamino)propyl]carbodiimide; Woodward's reagent K, *N*-ethyl-5-phenylisoxazolium-3'-sulfonate; NBD-Cl, 7-chloro-4-nitro-2,1,3-benzoxadiazole; PEI, poly(ethylenimine); EDTA, ethylenediaminetetraacetic acid; Tris-HCl, tris(hydroxymethyl)aminomethane hydrochloride; PPO, 2,5-diphenyloxazole; POPOP, 1,4-bis(5-phenyl-2-oxazolyl)benzene; PCA, perchloric acid; PEP, phosphoenolpyruvate; SDS, sodium dodecyl sulfate.

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syringes (1 cm^3) used in binding assays were purchased from Becton Dickinson Co. Radioactive [^{3}H]AMP-PNP and ^{57}Co were obtained from ICN Radiochemicals. [^{3}H]AMP-PNP was found to decompose slowly over a several week period and for this reason was never used after storage for more than 2 weeks. All other reagents were of the highest purity commercially available.

Methods

Purification of F_1-ATPase from rat mitochondria was carried out either by the procedure of Catterall and Pedersen (1971) as modified by Pedersen et al. (1981) or by the more recent, rapid $CHCl_3$ procedure of Williams et al. (1984b).

Assay for ATPase Activity. In assays, directed at determining the metal ion specificity of F_1, ATPase activity was assayed by following the release of inorganic phosphate using the procedure of Baginski et al. (1974). Prior to assay, purified F_1 stored in 250 mM KP_i + 5 mM EDTA was precipitated twice at room temperature with an ammonium sulfate solution containing 3.5 M ammonium sulfate, 1 mM EDTA, and 10 mM Tris-HCl, pH 7.5. Between precipitations, the enzyme was dissolved in 0.2 M K_2SO_4 and 10 mM Tris-HCl, pH 8.0. Centrifugation of the precipitate was performed at 18000*g* for 20 min. The ATPase assay mixture contained the following in a total volume of 1 mL: 4 μg of F_1, concentrations of nucleoside triphosphate specified in figures and tables, varying metal ion, and 50 mM Tris-HCl, pH 7.6. Incubations were terminated after 1 min at 25 °C by the addition of 0.1 mL cold 2.5 M perchloric acid. The 1-min time point lies well within the range of linearity of the assay under all conditions used in these studies. In all other ATPase assays, the spectrophotometric procedure (Catterall & Pedersen, 1971) was used in which ADP formed was coupled to the pyruvate kinase and lactate dehydrogenase reactions.

Separation of F_1-ATPase into an $\alpha\gamma$ fraction and a $\beta\gamma\delta\epsilon$ fraction was carried out exactly as described by Williams et al. (1984a) by placing 1.5 mg/mL F_1 in 250 mM KP_i + 5 mM EDTA, pH 7.5, at 0–4 °C for 16 h and then warming at 37 °C for 30 min prior to centrifugation.

Determination of the amount of magnesium that is tightly bound to isolated F_1-ATPase and subfractions thereof was carried out by using atomic absorption spectrometry. Prior to the determination, F_1 was precipitated twice with ammonium sulfate exactly as described under the ATPase assay procedure above. The final pellet was dissolved in 50 mM Tris-HCl buffer, pH 7.6, which had been treated previously by passing a 10× stock solution across a precycled Chelex column (Pasteur pipet filled with Chelex). The buffer was routinely stored in plastic vessels prewashed with 0.1 N nitric acid. F_1 contained in this metal ion free buffer was then chromatographed on a Sephadex G-25 column (0.9 × 23 cm) by using the same buffer. These preliminary treatments assure both that the enzyme contains no loosely bound metal and that the presence of contaminating metals within the buffer reagent is minimized.

Atomic absorption chromatography of F_1 treated in the above manner was carried out on a Perkin-Elmer 370 atomic absorption spectrometer at 285 nm using a Mg lamp and a slit width of 0.7. F_1 samples were always determined in parallel with elution buffer and a 1.17 μM Mg^{2+} standard. All sample determinations were performed in duplicate with three separate samples.

Determination of the amount of added Co^{2+} that binds to F_1-ATPase was determined by the centrifugation–column technique (Garrett & Penefsky, 1975). Prior to the binding assay, F_1, stored in 250 mM KP_i + 5 mM EDTA, pH 7.5, was precipitated once with ammonium sulfate exactly as described under the ATPase assay procedure above. The pellet was dissolved in 50 mM Tris-HCl, pH 7.6, and used directly in the binding assay. The binding assay mixture contained the following in a total volume of 100 μL: 5 mM $CoCl_2$ (0.2 μCi of $^{57}CoCl_2$), 50 mM Tris-HCl, pH 7.6, 125 μg of F_1, and where indicated 5 mM nucleotide. After incubation at 25 °C (see legends to tables for times), the entire reaction mixture was loaded onto a Sephadex G-50 "fine" column (1 cm^3 tuberculin syringe with a filter at the bottom) preequilibrated with 50 mM Tris-HCl, pH 7.6, and precentrifuged for 2 min at 2500 rpm in an IEC Model HN-SII clinical centrifuge. Centrifugation of the reaction mixture was carried out for 2 min at 2500 rpm to separate cobalt bound to the enzyme from the free metal ion. A control incubation without F_1 was always carried out to assure that "spillover" of the unbound metal did not elute with F_1. Radioactivity was assessed on the entire eluting fraction dissolved in 10 mL of a liquid scintillation cocktail containing 15.2 g of PPO, 0.38 g of POPOP in 3.8 L of toluene, and 2.28 L of ethanol.

Determination of the amount of nucleotide that is tightly bound to F_1-ATPase as isolated was carried out by using the luciferin–luciferase assay. Prior to assay, the enzyme was pretreated as indicated in the table legends and then dissolved in 2.5 M perchloric acid, incubated on ice for 15 min, neutralized with an equal volume of 2.5 M KOH, and centrifuged at 18000*g*. The luciferin–luciferase assay buffer (1 mL) consisting of 20 mM Hepes–NaOH, 10 mM KP_i, 5 mM $MgCl_2$, and 1 mM NaEDTA, pH 7.6, was first added to the reaction chamber of an LKB-Wallac Model 1250 luminometer (Turku, Finland). This was followed by respective 10-μL additions each of 2 mg/mL luciferase in 0.5 M sodium glycylglycine and 15 mM luciferin in 0.5 M sodium glycylglycine. For ATP determinations, an aliquot of the supernatant after PCA–KOH treatment was added and the luminescence monitored by using an LKB 2210 strip-chart recorder. For ADP determinations, an aliquot of the same supernatant was added to the assay medium containing in addition to the other compounds 0.1 mM PEP and 5 units of pyruvate kinase.

Determination of the amount of added nucleotide that binds to F_1-ATPase was carried out both by the column–centrifugation technique (Garrett & Penefsky, 1975) and by the equilibrium dialysis method described by Englund et al. (1969). The column centrifugation method was carried out exactly as described above with the indicated amounts of ^{3}H-nucleotide (0.05 μCi) replacing cobalt in the assay medium. Prior to assay, F_1 was precipitated the indicated number of times (see tables) with ammonium sulfate as described under the ATPase assay procedure above and dissolved in 50 mM Tris-HCl, pH 7.6. Assessment of nucleotide binding by equilibrium dialysis was carried out in lucite dialysis cells exactly like those designed by Englund et al. (1969). Each cell contained two pairs of sample compartments (30 μL each) separated by a semipermeable dialysis membrane. Dialysis membranes were prepared from Visking dialysis tubing (A. H. Thomas, inflated diameter = $^{27}/_{32}$ in.) which had been boiled in 2 large volumes of 5% Na_2CO_3–2% EDTA and stretched linearly and circularly by the technique of Craig and King (1962). The equilibration rate of ADP was increased approximately 3-fold by the stretching procedure. A 1-mm glass bead was inserted in each compartment to aid in mixing.

The experiment was initiated by adding 20 μL of enzyme solution to the "inside" cell compartment and 20 μL of a solution of adenine nucleotide containing about 70 000 cpm of ^{3}H-labeled adenine nucleotide to the "outside" cell com-

Table I: Magnesium and Nucleotide Bound to F_1-ATPase of Rat Liver As Isolated[a]

component assayed	mol/mol of F_1	Mg^{2+}/nucleotide ratio
Mg^{2+}	1.88	1.1
ATP + ADP	1.65	

[a] To assure that loosely bound ligands were removed from isolated F_1, the enzyme was precipitated with ammonium sulfate as described under Methods. Prior to magnesium determinations, the ammonium sulfate precipitated F_1 preparation was also chromatographed on a Sephadex G-25 column as described under Methods. Magnesium was determined by atomic absorption spectrometry and ATP + ADP by the luciferin–luciferase assay, both of which are described in detail under Methods. The values represent an average of three determinations on different F_1 preparations.

partment. Additions were made with a 25-μL Hamilton syringe with a blunt needle point. Cells were then sealed with tape and incubated for 3 h at room temperature while being rotated at 4 rpm about the long axis of the cell. After dialysis, one 15-μL aliquot was removed from each inside compartment. Protein concentration and radioactivity were determined on aliquots of this solution. One 15-μL aliquot was removed from each outside compartment and pipetted into 100 μL of H_2O. Radioactivity was determined on aliquots of this solution. Recoveries of protein, total radioactivity, and radioactivity in the labeled compound were quantitative.

The concentration of free ligand (L) was calculated from the radioactivity in the outside compartment. The concentration of bound ligand was calculated from the difference in radioactivity between the two compartments. The average number of ligand molecules bound per molecule of enzyme (r) was calculated from the concentration of bound ligand and the input protein concentration. The data were then plotted as r/L vs. r as recommended by Scatchard (1949). If the binding is to a single set of n independent binding sites of dissociation constant K_{diss}, the data fall on a straight line described by the following equation: $r/L = (n/K_{diss}) - (r/K_{diss})$. If the binding is to multiple sets of sites or to a set of interacting sites, the data fall on a curved plot.

Determination of Protein. Protein was determined either by the method of Bradford (1976) or by using the method of Lowry et al. (1951) following precipitation of the protein with 5% trichloroacetic acid. Bovine serum albumin was used as standard. The two methods gave essentially identical results (within 10%) for rat liver F_1. The molecular weight of F_1 used in all stoichiometry calculations was 384 000 (Catterall & Pedersen, 1971).

Results

Enzyme History and Properties. F_1 preparations used for these studies, whether prepared by the more recent chloroform procedure (Williams et al., 1984b) or by the earlier procedure of Catterall and Pedersen (1971), have been shown to be competent in restoring almost fully the rate of ATP synthesis to F_1-depleted inner membrane vesicles (Williams et al., 1984b; Pedersen & Hullihen, 1978). Such preparations when assayed in Tris-HCl buffer, pH 7.5 and 25 °C, exhibit an ATPase specific activity of between 25 and 35 μmol of ATP hydrolyzed min^{-1} mg^{-1}. They show five subunit bands upon SDS–polyacrylamide gel electrophoresis in the stoichiometric ratio $\alpha_3\beta_3\gamma\delta\epsilon$ (Catterall et al., 1973).

Magnesium and Nucleotide Bound to Rat Liver F_1 As Isolated. Results presented in Table I show that 1 mol of rat liver F_1 as isolated contains 1.88 mol of Mg^{2+} and 1.65 mol of nucleotide. The Mg^{2+}/nucleotide ratio is, therefore, very close to 1. Although it is possible that the bound Mg^{2+} and nucleotide are associated, nucleotide was found to be rather

Table II: Effect of Various Treatments on Amount of Magnesium Tightly Bound to F_1-ATPase[a]

treatment	mol of Mg^{2+}/mol of F_1
(1) F_1	1.89
(2) F_1 incubated at RT, 1 h, in 50 mM Tris-HCl, 50 mM EDTA, pH 7.6	1.06
(3) F_1 incubated overnight, 0–4 °C, in 250 mM KP_i, 50 mM EDTA, pH 7.6	1.06
(4) F_1 incubated overnight, RT, in 50 mM Tris-HCl, 50 mM EDTA, pH 7.6	0.36

[a] The purified enzyme was precipitated twice with ammonium sulfate and chromatographed on a Sephadex G-25 column exactly as described under Methods. Where indicated, the enzyme was subjected to further treatments after the ammonium sulfate precipitation steps and prior to Sephadex G-25 chromatography. In each case, the enzyme was processed and subjected to atomic absorption spectrometry exactly as described under Methods. Values represent averages of duplicate determinations on three different F_1 preparations. RT, room temperature.

easy to remove by a variety of procedures including cold denaturation, repeated ammonium sulfate precipitation, and incubation in 50 mM Tris-HCl buffer, pH 7.5, followed by column centrifugation (data not shown). As will be noted below, bound Mg^{2+} is more resistant to such treatments, indicating a direct association with F_1.

Effect of Various Treatments on the Amount of Magnesium Tightly Bound to Rat Liver F_1 As Isolated. Results summarized in Table II show that both moles of Mg^{2+} bound to isolated F_1 remain bound after the enzyme has been precipitated twice with ammonium sulfate and then chromatographed on a Sephadex G-25 column in a buffer containing 50 mM Tris-HCl, pH 7.6. Significantly, 1 mol of Mg^{2+}/mol of F_1 can be removed by incubating F_1 at room temperature (23–25 °C) for 1 h in 50 mM Tris–50 mM EDTA, pH 7.6, followed by Sephadex G-25 chromatography. The same result is obtained by incubating F_1 overnight at 0–4 °C in 250 mM KP_i–50 mM EDTA, pH 7.6, prior to the gel filtration step. In both cases, approximately 1 mol of Mg^{2+}/mol of F_1 remains firmly bound to F_1. About 69% of this very tightly bound metal can be removed by incubating the enzyme at room temperature overnight in 50 mM Tris-HCl–50 mM EDTA, pH 7.6, prior to gel filtration chromatography. However, these conditions also result in noticeable enzyme precipitation.

Effect of Cold Denaturation–Rewarming Cycle on the 1 mol of Mg^{2+} Very Tightly Bound to 1 mol of F_1. We recently reported that when rat liver F_1 is subjected to a cold denaturation–rewarming cycle under defined conditions, the enzyme can be easily separated into $\alpha\gamma$ fraction (precipitate) and a $\beta\gamma\delta\epsilon$ fraction (supernatant) (Williams et al., 1984a). A significant amount of the $\alpha\gamma$ pair remains associated upon gel filtration chromatography in the presence of dithiothreitol, indicating that the γ subunit may interact with at least one α subunit in the F_1 complex (Williams et al., 1984a). When this cold denaturation–rewarming cycle was used to prepare $\alpha\gamma$ and $\beta\gamma\delta\epsilon$ fractions in this study, it was found that about 80% of the very tightly bound Mg^{2+} remained associated with the $\alpha\gamma$ fraction (Table III). These studies, together with those summarized in Table II, indicate that of the 2 mol of Mg^{2+} tightly associated with 1 mol of isolated F_1, one is more tightly bound than the other. In addition, they indicate that the tight metal binding site may lie on either an α or an γ subunit (or at an interface thereof).

Use of Co^{2+} and AMP-PNP To Detect Additional Divalent Cation and Nucleotide Binding Sites on F_1. The studies described above show quite clearly that isolated F_1 from rat

Table III: Relative Distribution of Very Tightly Bound Magnesium in $\alpha\gamma$ and $\beta\gamma\delta\epsilon$ Fractions of F_1-ATPase after Cold Dissociation[a]

treatment	mol of Mg^{2+}/mol of F_1 (or part)
F_1 incubated overnight at 0–4 °C in 250 mM KP_i, 50 mM EDTA, not separated	1.06
precipitate derived from the above after warming ($\alpha\gamma$ fraction)	0.89
supernatant derived from the above ($\beta\gamma\delta\epsilon$ fraction)	0.32

[a] F_1 was precipitated once with ammonium sulfate exactly as described under Methods and then dissolved at 1.5 mg/mL in 250 mM KP_i–5 mM EDTA, pH 7.5. It was then subjected to cold treatment and rewarming to separate the $\alpha\gamma$ fraction from the $\beta\gamma\delta\epsilon$ fraction. See Methods for details. The samples were then processed for atomic absorption spectrometry exactly as described under Methods. Values represent averages of duplicate determinations on at least three different F_1 preparations.

Table IV: Relative Capacities of Divalent Cations Other than Magnesium To Support ATPase Activity Catalyzed by F_1[a]

metal ion in assay	ATP hydrolytic act. (μmol min^{-1} mg^{-1})
Mg^{2+}	34
Co^{2+}	35
Zn^{2+}	53
Mn^{2+}	55

[a] Purified F_1-ATPase was precipitated twice with ammonium sulfate and then assayed for ATPase activity exactly as described under Methods. ATP and divalent cation were each present at a concentration of 2.5 and 1 mM, respectively. Values represent averages obtained of duplicate determinations performed on two different F_1 preparations.

liver, which is fully competent in restoring ATP synthesis to F_1-depleted inner membrane vesicles (Williams et al., 1984b; Pedersen & Hullihen, 1978), contains at least two ligand binding domains. In order to establish how many additional ligand binding domains reside on the isolated enzyme, we used Co^{2+} as a divalent cation and AMP-PNP as a nucleotide analogue. Co^{2+} was used in place of Mg^{2+} because ^{57}Co, in contrast to Mg^{2+}, can be obtained as a weak γ emitter with a relatively long half-life. Justification of its use is presented in Table IV where it is shown that Co^{2+}, in contrast to the "activating" cations Zn^{2+} and Mn^{2+}, gives an ATPase specific activity value very near that observed with Mg^{2+}. Justification of the use of AMP-PNP was based on data not presented here which showed that this ATP analogue is a potent competitive inhibitor relative to ATP in the ATPase reaction catalyzed by F_1. The apparent K_m of rat liver F_1 for ATP is 1.1 mM whereas the apparent K_i for AMP-PNP was found to be 2.2 μM.

Cobalt Binding to F_1 in the Presence and Absence of AMP-PNP and Other Nucleoside Triphosphates. Results presented in Table V show that in the absence of added nucleotide and the presence of 5 mM $CoCl_2$, rat liver F_1 incorporates only about 1 mol of Co^{2+}/mol of F_1. When 5 mM AMP-PNP is included in the binding assay, a total of 2.8 mol of Co^{2+}/mol of F_1 is incorporated. Significantly, the hydrolytic substrates ATP, GTP, and ITP (Pedersen, 1976) are quite effective in replacing AMP-PNP in the binding assay.

Results presented in Table VIA show that when 2.8 mol of Co^{2+} is bound to F_1 in the presence of AMP-PNP the very tight Mg^{2+} binding site remains intact. However, Mg^{2+} bound at the second site has been displaced by Co^{2+} or most likely by an AMP-PNPCo complex (see below).

These results show that rat liver F_1 can retain almost 4 mol of divalent cation provided AMP-PNP or a hydrolytic triphosphate substrate is present.

Table V: Binding of Co^{2+} to F_1-ATPase in the Absence and Presence of Nucleoside Triphosphates[a]

assay components	mol of Co^{2+}/mol of F_1
F_1 + Co^{2+}	1.1
F_1 + Co^{2+} + AMP-PNP	2.8
F_1 + Co^{2+} + ATP	2.4
F_1 + Co^{2+} + GTP	2.2
F_1 + Co^{2+} + ITP	2.3

[a] Binding assays were carried out for 30 min with either 5 mM $^{57}CoCl_2$ or 5 mM $^{57}CoCl_2$ + 5 mM nucleoside triphosphate exactly as described under Methods. Values represent the average of four different experiments carried out in duplicate on different F_1 preparations.

Table VI: Experiments Which Show That (A) 1 mol of Very Tightly Bound Mg^{2+} Remains Bound When AMP-PNP Supports the Binding of Almost 3 mol of Co^{2+}/mol of F_1 and (B) a Nearly Equivalent Amount of AMP-PNP (to That of Co^{2+}) Binds to F_1 with or without Added Co^{2+}[a]

Experiment A assay components	mol of Co^{2+}/mol of F_1	mol of Mg^{2+}/mol of F_1
F_1 + AMP-PNP + Co^{2+}	2.8	0.92

Experiment B assay components	mol of AMP-PNP/mol of F_1
F_1 + AMP-PNP	2.3
F_1 + AMP-PNP + Co^{2+}	2.8

[a] In experiment A, F_1-ATPase was loaded with Co^{2+} in the presence of AMP-PNP as described in Table V and in more detail under Methods. The enzyme was then processed for atomic absorption spectrometry also as described under Methods. Atomic absorption spectrometry was carried out to determine the amount of bound magnesium exactly as described under Methods. Values represent averages obtained from four different F_1 preparations. In experiment B, binding assays were carried out for 30 min in the presence of 5 mM [^{3}H]-AMP-PNP followed by column centrifugation as described under Methods. Where indicated, 5 mM $CoCl_2$ was present. Values represent averages of five different experiments on five different F_1 preparations.

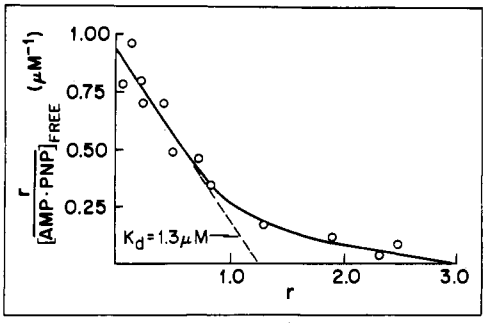

FIGURE 1: Scatchard plot (1949) summarizing the equilibrium binding of AMP-PNP to rat liver F_1. Equilibrium dialysis was carried out exactly as described under Methods. The outside compartment contained the following in 20 μl: 0.5 mM $MgCl_2$, 0.5 mM EDTA, 10 mM Tris-HCl, pH 7.6, and in various experiments AMP-PNP concentrations ranging from 0.1 to 72 μM. The inside compartment contained 50 μg of F_1 in 20 μL of 0.1 M KCl. F_1 has been precipitated once with 3.5 M $(NH_4)_2SO_4$–10 mM Tris-HCl, pH 7.5.

AMP-PNP Binding to F_1 in the Presence and Absence of Co^{2+}.[2] Results summarized in Table VIB show that, under

[2] In control studies, fresh AMP-PNP (Sigma) migrated as a single spot on PEI–cellulose (solvent = 1 M LiCl + 2 M HCOOH). After incubation with rat liver F_1-ATPase under conditions identical with those used in binding assays, AMP-PNP migrated again as a single spot. The solvent system used clearly separated a control mixture of 20 nmol each of ATP, AMP-PNP, ADP, and AMP which could be readily detected under ultraviolet light. Therefore, AMP-PNP does not appear to undergo significant hydrolysis under the conditions of the experiments described here.

Table VII: ADP Binding to F_1 in the Absence and Presence of AMP-PNP[a]

assay components	nucleotide concn (mM)	mol of ADP/mol of F_1	mol of AMP-PNP/mol of F_1
F_1 + ADP[b]	5	1.0	
F_1 + ADP[b]	0.3	0.99	
F_1 + ADP[b] + AMP-PNP	both 5	1.2	
F_1 + AMP-PNP[b]	5		2.7
F_1 + AMP-PNP[b] + ADP	both 5		2.6

[a] Binding assays were carried out for 20 min in the presence of 5 mM Co^{2+} using either [^{3}H]ADP or [^{3}H]AMP-PNP at the concentrations indicated. Column centrifugation was then carried out as described under Methods. All experiments represent averages of duplicate determinations on two different F_1 preparations. [b] Radioactive species.

Table VIII: Comparison of the Effect of NBD-Cl on the Binding of Various Ligands to F_1[a]

ligand assayed	mol/mol of F_1 before NBD-Cl	mol/mol of F_1 after NBD-Cl[b]	% inhibition
Co^{2+} bound with AMP-PNP	2.7	1.1	60
Co^{2+} bound without AMP-PNP	0.8	1.2	0
AMP-PNP bound (Co^{2+} present)	2.9	0	100
ADP bound (Co^{2+} present)	1.0	0.5	50

[a] Where indicated, Co^{2+}, AMP-PNP, ADP, and NBD-Cl were present at 5 mM concentration. Prior incubation of F_1 with 5 mM NBD-Cl was carried out for 20 min in 50 mM Tris-HCl buffer, pH 7.6, 25 °C. Binding of the various ligands was assessed by the column centrifugation procedure (see Methods). Values represent averages of four different experiments. [b] ATPase activity of F_1 is completely inhibited as assessed by the spectrophotometric assay (see Methods).

the same conditions which support the binding of 2.8 mol of Co^{2+}/mol of F_1 (see Tables V and VIA), a comparable amount of AMP-PNP binds as well. However, in contrast to Co^{2+} binding, which requires AMP-PNP for maximal binding to rat liver F_1 (Table V), maximal binding of AMP-PNP occurs independently of the presence of divalent cation. These data indicate that Co^{2+} is brought to F_1 via an AMP-PNPCo complex.

Results presented in Figure 1 show that about 3 mol of AMP-PNP binds to 1 mol of rat liver F_1 using a completely separate binding assay. In contrast to the above results which were obtained at 5 mM AMP-PNP (±5 mM Co^{2+}) using the column centrifugation method, results obtained in Figure 1 were obtained at low AMP-PNP and Mg^{2+} concentrations (<0.1 mM) by using an equilibrium binding assay (see methods). A Scatchard analysis of the binding data shows that of the nearly 3 mol of AMP-PNP which binds to F_1, one binds more tightly than the other two, exhibiting a dissociation constant of about 1.3 μM. It seems likely that this tight site represents the same site noted above which bound AMP-PNPCo while displacing the exchangeable Mg^{2+}.

Finally, in experiments not reported here, we were unable to detect additional AMP-PNP binding sites on F_1 when incubation times as long as 2 h were employed or when the temperature was increased from 25 to 37 °C.

ADP Binding to F_1 in the Presence and Absence of AMP-PNP. Previous studies from this laboratory (Catterall & Pedersen, 1972; Pedersen, 1975) have shown that rat liver F_1 contains only a single ADP binding site that can be detected with or without Mg^{2+} by using an equilibrium dialysis assay. This site has a dissociation constant of about 1 μM and is highly specific for ADP and dADP. Consistent with these earlier findings, results presented in Table VII show that rat liver F_1 binds per mole only 1 mol of ADP also when the column centrifugation method is used. Significantly, in the experiment shown, 5 mM ADP and 5 mM metal were used to assure that any accessible ADP sites were fully saturated. As in the earlier studies (Catterall & Pedersen, 1975; Pedersen, 1975), added divalent cation is not essential and can be omitted without altering ADP binding.

Experiments also presented in Table VII show that AMP-PNP concentrations as high as 5 mM are without effect on ADP binding. Likewise, ADP concentrations as high as 5 mM have no effect on the binding of AMP-PNP. In both cases, the two nucleotides bind exclusively of the other with a total of four nucleotide binding sites being loaded, almost three with AMP-PNP and one with ADP. (Under these conditions, there are no additional tightly bound nucleotides. They are removed during the column centrifugation.)

Effect of Covalent Labeling Agents on Divalent Cation and/or Nucleotide Binding to F_1. Experiments were carried out to assess the effects of a variety of covalent labeling agents on ligand binding to rat liver F_1. Agents such as those which exhibit specificity for carboxyl groups (DCCD, EDAC, and Woodward's reagent), arginine residues (2,3-butanedione and phenylglyoxal), and histidine residues (diethyl pyrocarbonate) are without effect on Co^{2+} binding to F_1 supported by AMP-PNP (data not shown). Surprisingly, even when incubated with rat liver F_1 in 50 mM Tris-HCl, pH 7.6 for 20 min at high concentrations (≤5 mM), these agents exhibit little inhibitory effect (<30%) on ATPase activity of the enzyme. In contrst, NBD-Cl, a purported tyrosine labeling agent of F_1 at pH 7.6 (Andrews et al., 1984; Ferguson et al., 1974), competely inhibits ATPase activity at 5 mM and prevents Co^{2+} binding at two of the three binding sites (Table VIII). A third Co^{2+} binding domain remains resistant to NBD-Cl (Table VIII). As emphasized above, AMP-PNP "brings to" one molecule of F_1 two of the three Co^{2+} molecules whereas a third AMP-PNPCo appears to displace Mg^{2+} at one of the two Mg^{2+} binding sites (Table V). It seems likely, therefore, that the NBD-Cl-resistant site is identical with the exchangeable Mg^{2+} site. Additional evidence for this view is derived from data presented in Table VIII where it is shown that NBD-Cl is without effect on the single Co^{2+} site observed when AMP-PNP is absent.

Results presented in Table VIII show also that 5 mM NBD-Cl completely prevents binding at all three AMP-PNP binding sites while only partially inhibiting binding at the ADP binding site. These findings provide further evidence that the three AMP-PNP binding domains on rat liver F_1 are separate and distinct from the ADP binding domain.

Discussion

Results of experiments described here show that rat liver F_1 contains a minimum of two divalent metal binding sites (Tables I and II) and four nucleotide binding sites (Table VII) where binding is directly to F_1. Three of the nucleotide binding sites can be filled with AMP-PNP (Tables VIA and VII) whereas a fourth site is specific for ADP (Table VII). In the presence of AMP-PNP, two additional metal ions bind to F_1 (Table V), but in this case are brought to the enzyme by AMP-PNP (compare Tables V and VIB). Overall, the data are consistent with an F_1 molecule which can bind one molecule of Mg^{2+} at a nonexchangeable site, another molecule of Mg^{2+} (or AMP-PNPMg) at an exchangeable but high-affinity site (K_d = 1.3 μM), two molecules of AMP-PNPMg at lower affinity sites, and a single molecule of ADP at a

high-affinity site [$K_d \simeq 1$ μM (Catterall & Pedersen, 1972)] distinct from the AMP-PNP sites.

To reconcile these binding data with the unusual structure of rat liver F_1 which contains only a single copy of the three smaller subunits $\gamma\delta\epsilon$ per three $\alpha\beta$ pairs (Catterall et al., 1973), the model presented in Figure 2A is proposed. The model depicts the very tight (nonexchangeable) Mg^{2+} site, the exchangeable Mg^{2+} site (occupied as an $\overline{Mg\text{–}ATP}$ complex), and the ADP binding site as residing on an asymmetric $\alpha\beta$ pair tagged by one (or more) of the smaller subunits. Two additional nucleotide binding sites, occupied as E–NTPMg complexes, are ascribed to two pure $\alpha\beta$ pairs. The latter are depicted as NTPMg rather than ATPMg as experiments described herein show that the hydrolytic substrates ATP, ITP, and GTP will replace AMP-PNP in supporting near-maximal metal binding to rat liver F_1 (Table V).

Studies reported here on metal binding stoichiometry are in agreement with those reported earlier both by Senior's laboratory (Senior, 1979b, 1981; Senior et al., 1980) for the bovine heart F_1 and, more recently, by the laboratory of Schuster (Daggett et al., 1985) on the same enzyme. Senior and co-workers (Senior, 1979b, 1981; Senior et al., 1980) demonstrated the presence of one nonexchangeable and one exchangeable metal binding site on the heart enzyme whereas Schuster and co-workers (Daggett et al., 1985) showed that two additional metals per molecule of F_1 could be detected in the presence of AMP-PNP. It is important to point out, however, that in neither of these studies was the binding of nucleotide monitored in the absence and presence of metal. As emphasized in the present report, the two extra metal ions associated with F_1 in the presence of AMP-PNP are brought in with the nucleotide, whereas a third AMP-PNP–metal complex exchanges with one of the two endogenously bound Mg^{2+} ions (Tables V and VIA). Significantly, AMP-PNP binds to rat liver F_1 at near-maximal levels ($\sim$2.3 mol/mol of F_1) in the absence of added metal (Table VIB), indicating a direct association of nucleotide with the enzyme at the three nucleoside triphosphate binding sites.

The data obtained showing that rat liver F_1 contains three binding sites for AMP-PNP which are detected by equilibrium binding measurements (Table VIB and VII, Figure 1) and that one of the three is of higher affinity than the other two (Figure 1) are in general agreement with the work of Cross and Nalin (1982) on the bovine heart enzyme. It is important to point out, however, that the liver enzyme binds these nucleotides with much less affinity than does the heart enzyme. Thus, the highest affinity AMP-PNP site on the liver enzyme binds this nucleotide with a K_d of about 1 μM (Figure 1) whereas the highest affinity AMP-PNP site on the heart enzyme binds AMP-PNP with a K_d of 18 nM (Cross & Nalin, 1982).

The finding that the liver enzyme, even when depleted of endogenous nucleotides binds only 3 mol of AMP-PNP and 1 mol of ADP may appear at first to be in disagreement with studies on the heart enzyme where two additional binding sites can be detected (Harris et al., 1973; Cross & Nalin, 1982; Weber et al., 1985). It should be noted, however, that the heart enzyme is product-inhibited by ADP with a K_i of 30 μM (Hammes & Hilborn, 1971) an order of magnitude less than the liver enzyme which exhibits a K_i of about 300 μM (Catterall & Pedersen, 1974). Therefore, it seems likely that the liver enzyme may contain additional nucleotide binding sites associated with product inhibition that cannot be readily detected by equilibrium binding assays. Along the lines of this discussion, it seems important to note that Gautheron and her colleagues (Gautheron et al., 1984) have reported that pig heart F_1 contains a single site involved in ADP inhibition of the ATPase activity of the enzyme. Using an affinity label of ADP, these workers have localized this site on a β subunit

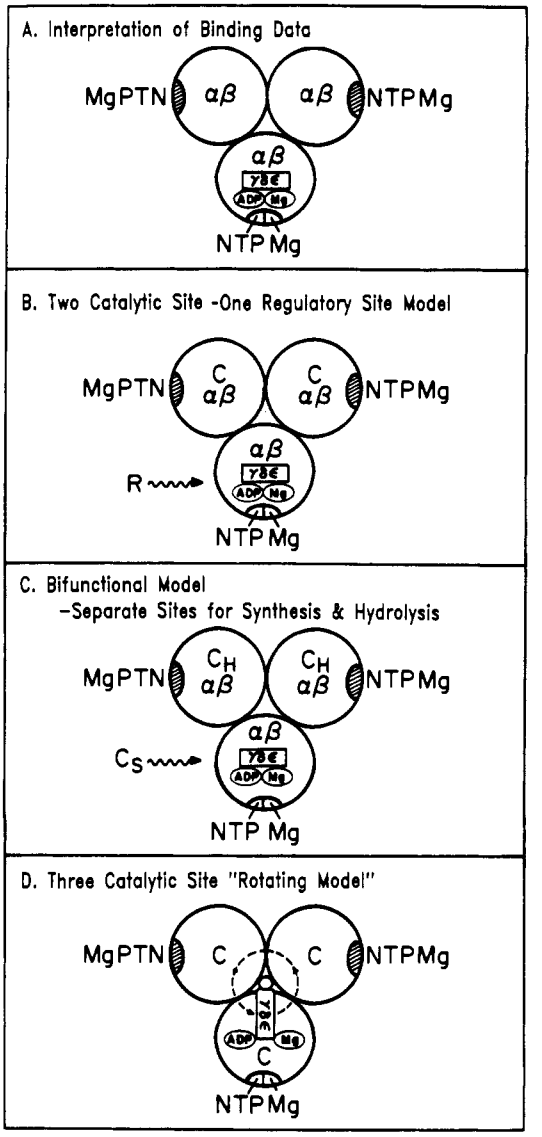

FIGURE 2: (A) Interpretation of ligand binding data obtained for rat liver F_1. As rat liver F_1 consists of a single copy of the smaller subunits $\gamma\delta$ and ϵ, per three $\alpha\beta$ pairs, the structure of the enzyme is depicted as being asymmetric with the smaller subunits interacting with only one $\alpha\beta$ pair [see Williams et al. (1984a) for structural evidence for one of the three $\alpha\beta$ pairs being "tagged" by one or more of the smaller subunits]. The binding data presented here are best interpreted within the framework of this structural model and, in fact, provide additional support for it. Thus, the single tight Mg^{2+} site, the single exchangeable Mg^{2+} site (present as an $\overline{NTP\text{–}Mg}$ complex), and the single ADP binding site are best depicted as interacting with the single asymmetric or tagged $\alpha\beta$ pair. The pure $\alpha\beta$ pairs are depicted as binding the other two nucleotides as E–NTPMg complexes. These binding data are discussed in the text with respect to their capacity to be accommodated by three current models for the function of F_1: (B) *a two catalytic site–one regulatory site model* where the tagged $\alpha\beta$ pair is a regulatory subunit and the pure $\alpha\beta$ pairs are catalytic subunits; (C) *a bifunctional model* where the tagged $\alpha\beta$ pair, C_S, preferentially catalyzes ATP synthesis and the pure $\alpha\beta$ pairs, C_H, preferentially catalyze ATP hydrolysis; (D) *a three catalytic site rotating model* where each subunit is directed in sequence by the tag to be catalytic. The latter may occur by rotation of one or more of the smaller subunits about the larger subunits or vice versa. The single high-affinity ADP and single Mg^{2+} sites in this model must either be associated directly with the tag or become unmasked on each of the $\alpha\beta$ pairs upon interaction with the tag.

which they suggest must be unequivalent to the other two β subunits.

Studies with covalent labeling agents show that rat liver F_1, in 50 mM Tris-HCl, pH 7.6, is inhibited less than 35% by high concentrations of agents which exhibit specificity for carboxyl groups, arginine residues, and histidine residues. Under these conditions, only NBD-Cl markedly inhibits ATPase activity, providing 100% inhibition at a concentration of 5 mM. Significantly AMP-PNP binding is also completely inhibited under these conditions, while one of the three Co^{2+} binding sites remains resistant (Table VIII). Binding of ADP is inhibited by only 50%. These data provide further support for the model proposed in Figure 2A which depicts one of the three NTPMg sites on rat liver F_1 as different from the other two and in addition depicts the single ADP site as separate and distinct from the AMP-PNP sites.

In concluding this discussion, it seems important to focus briefly on current models (Gresser et al., 1982; Cox et al., 1984; Pedersen & Amzel, 1985; Wang, 1985; Mitchell, 1985) for the function of F_1-ATPases and the capacity of binding data obtained here for the rat liver enzyme to be accommodated by one or more of these models. One model (Figure 2B) for the function of F_1 depicts the enzyme as containing one regulatory $\alpha\beta$ pair (the pair tagged by one or more of the smaller subunits) and two catalytic units, where either the β subunit or the $\alpha\beta$ pairs comprise the catalytic sites (Gautheron et al., 1984; Pedersen & Amzel, 1985). Catalytic sites in this case can be either hydrolytic or synthetic. A second or "bifunctional" model (Figure 2C) depicts the tagged $\alpha\beta$ pair as being specialized preferentially for ATP (or NTP) synthesis with the two pure $\alpha\beta$ pairs being specialized preferentially for NTP hydrolysis (Pedersen & Amzel, 1985). Finally, a third model (Figure 2D) depicts F_1 as consisting of three potential catalytic $\alpha\beta$ pairs, each of which must be "directed" to become catalytic in a sequential manner as the tag (smaller subunits) rotates about the three $\alpha\beta$ pairs or the latter rotate about the tag (Gresser et al., 1982; Cox et al., 1984; Mitchell, 1985; Pedersen & Amzel, 1985). Assuming there was no rotation of the tag (or about the tag), the latter model would reduce to still a fourth model suggested by Wang (1985) which depicts the enzyme as consisting of one active catalytic site and two interacting regulatory sites.

As noted in Figure 2B–D, ligand binding data obtained for F_1 can be accomodated quite readily by each of the above models, because each model takes into account that one of the three $\alpha\beta$ pairs, either in a static enzyme (models in Figure 2B,C) or in an dynamic enzyme (model 2D), must be unequivalent to the other two. The single unequivalent $\alpha\beta$ pair is the obvious choice for the single tight Mg^{2+} binding site, the single exchangeable Mg^{2+} (or NTPCo) binding site, and the single high-affinity ADP binding site reported here for the rat liver enzyme. The additional two $\alpha\beta$ pairs can be assigned readily to the other two nucleotide–metal complexes that bind to rat liver F_1, in this case with nucleotide binding to the enzyme directly bringing the metal with it.

Further work is currently under way in this laboratory to distinguish among these three models or to determine whether another hitherto unproposed model can best account for the complex binding and kinetic data of this structurally unique enzyme.

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Kinetics and Mechanism of Human Leukocyte Elastase Inactivation by Ynenol Lactones†

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ABSTRACT: Human leukocyte elastase (HLE), a serine protease involved in inflammation and tissue degradation, can be irreversibly inactivated in a time- and concentration-dependent manner by ynenol lactones (**1–4**). Ynenol lactones that are α-unsubstituted do not inactivate but are alternate substrate inhibitors that are hydrolyzed by the enzyme. Ynenol lactones that are both substituted α to the lactone carbonyl and unsubstituted at the acetylene terminus are rapid inactivators of HLE and inactivate pancreatic elastase and trypsin more slowly. 3-Benzyl-5(*E*)-(prop-2-ynylidene)tetrahydro-2-furanone inactivates HLE with biphasic kinetics and an apparent second-order rate of up to 22 000 M^{-1} s^{-1} (pH 7.8, 25 °C). The rate of inactivation is pH-dependent and is slowed by a competitive inhibitor. The partition ratio is 1.6 ± 0.1. Rapid removal of ynenol lactone during the course of inactivation yields a mixture of acyl and inactivated enzyme species, which then shows a partial recovery of activity that is time- and pH-dependent. Inactivation is not reversible with hydroxylamine. The enzyme is not inactivated if the untethered allenone is added exogenously. All of these results are consistent with a mechanism involving enzyme acylation at serine-195 by the ynenol lactone, isomerization of the acyl enzyme to give a tethered allenone, and capture of a nucleophile (probably histidine-57) to inactivate the enzyme. Substitution at the acetylene terminus of ynenol lactones severely reduces their ability to inactivate HLE, because allenone formation is slowed and/or nucleophile capture is hindered. Chemical competence of each of these steps has been demonstrated [Spencer, R. W., Tam, T. F., Thomas, E. M., Robinson, V. J., & Krantz, A. (1986) *J. Am. Chem. Soc. 108*, 5589–5597].

The design and characterization of serine protease inhibitors, especially inhibitors of human leukocyte elastase (HLE),[1] is an active area of research since such compounds may have therapeutic utility in a number of degradative diseases (Starkey, 1977; Barrett, 1980). Inhibition by a variety of small molecules (i.e., excluding natural or recombinant polypeptide inhibitors) is known and may be subdivided into three mechanistic classes: (1) inhibition that is reversible with respect to both the inhibitor and the enzyme, (2) inhibition that is reversible with respect to the enzyme but in which the inhibitor is permanently altered (i.e., the inhibitor is actually a substrate), and (3) inhibition that is irreversible with respect to both the enzyme and the inhibitor. Table I expands on this classification with specific examples. Fully reversible inhibitors (class 1 above) include noncovalent and carbonyl agents. Inhibitors that form acyl enzymes belong in class 2,[2] though in some cases deacylation is so slow that the inhibition is, for practical purposes, irreversible. Alkylating agents are generally fully irreversible (class 3) in their action.

Known alkylating inhibitors may be further classified as those that are intrinsically electrophilic and those in which the electrophilic functionality is initially masked and exposed only

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[1] Abbreviations: HEPES, *N*-(2-hydroxyethyl)piperazine-*N*′-2-ethanesulfonic acid; HLE, human leukocyte elastase; HSE, human sputum elastase; MES, 2-(*N*-morpholino)ethanesulfonic acid; Me_2SO, dimethyl sulfoxide; PPE, porcine pancreatic elastase; SAAPVC, 7-(methoxysuccinylalanylalanylprolylvalinamido)-4-methylcoumarin; SAAPVFC, 7-(methoxysuccinylalanylalanylprolylvalinamido)-4-(trifluoromethyl)coumarin; TAPS, 3-[[tris(hydroxymethyl)methyl]amino]-propanesulfonic acid; TMAC, 7-[(trimethylacetyl)oxy]-4-methylcoumarin (see Materials and Methods).

[2] The lactones of Tobias et al. (1969) and Izbicka and Bolen (1981) are an interesting exception. Though these compounds acylate chymotrypsin, they can deacylate intramolecularly to regenerate the initial inhibitor.

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