Peptide Analogs of the Beef Heart Mitochondrial F_1-ATPase Inhibitor Protein

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ABSTRACT: Peptide analogs which correspond to the conserved region of the natural ATPase inhibitor protein from beef heart, *Candida utilis*, and *Saccharomyces cerevisiae* mitochondria were synthesized by solid-phase methodologies and tested for ATPase inhibitory activity. These peptides were found to be potent inhibitors of F_1-ATPase-catalyzed ATP hydrolysis in acidic reaction media, having I_{50} values of 1.1 ± 0.4 μM, 10 ± 5 μM, and 48 ± 19 μM, respectively. These results closely match those obtained for the naturally occurring inhibitor proteins. Additional peptides that correspond to the beef heart β-subunit near the binding site of the beef heart inhibitor protein and that possess a substantial homology with the conserved region of the inhibitor protein were synthesized. Several of these peptides were found to be inhibitors of the ATPase activity. The best inhibitor, with an I_{50} value of 20 ± 3 μM, was the peptide resembling the beef heart β-subunit comprising amino acids 394–413. This peptide most closely resembles the peptides derived from the conserved region of the inhibitor protein. The insertion of five glycine residues between the charge clusters in the β-394–413 peptide resulted in a peptide which was able to stimulate the hydrolysis of ATP.

The natural ATPase inhibitor protein (F_1I) was first isolated from beef heart mitochondria (Pullman & Monroy, 1963; Horstman & Racker, 1970), with similar subunits being isolated from rat liver (Chan & Barbour, 1976; Cintron & Pedersen, 1979), rat skeletal muscle (Yamada et al., 1980; 1981), and yeast mitochondria (Satre et al., 1975; Ebner & Maier, 1977). The inhibitor proteins from these species are able to cross-react with the mitochondrial F_1-ATPases isolated from the other species (Satre et al., 1975; Chan & Barbour, 1976; Cintron & Pedersen, 1979; Klein et al., 1977; Dianoux & Hoppe, 1987). The sequences of the inhibitor proteins from *Candida utilis* (Dianoux and Hoppe, 1987), *Saccharomyces cerevisiae* (Matsubara et al., 1981), and beef heart (Runswick et al., 1986; Walker et al., 1987) have been determined, and a region near the amino terminus of these peptides is highly conserved (Dianoux & Hoppe, 1997).

The ability of these analogous inhibitor proteins to inhibit the F_1-ATPase activity from the other species (Satre et al., 1975; Chan & Barbour, 1976; Cintron & Pedersen, 1979; Klein et al., 1977; Dianoux & Hoppe, 1987) led to the proposal that this conserved region may be involved in the interaction of the inhibitor protein with the F_1-ATPase. This is further supported by the observation that the peptides F_1I 10–88 and 16–84 derived from proteolytic digestions (Dianoux et al., 1981, 1982) of the beef heart F_1I are still active inhibitors of ATPase activity. The involvement of this conserved region in the inhibitory activity is also supported by studies using site-specific antibodies which bind to F_1I (Husain et al., 1985; Dreyfus et al., 1981; Audinet et al., 1986). However, peptides corresponding to the conserved region alone have not been shown to exhibit inhibitory activity.

The inhibition of ATPase activity results from the binding of a single F_1I to a single β-subunit of the F_1-ATPase complex in either the soluble or the membrane-bound form (Klein et al., 1980, 1981; Jackson & Harris, 1988). A fragment of the β-subunit, β-394–495, was shown to cross-link to F_1I during ATPase inhibition (Jackson & Harris, 1988). A comparison of the beef heart F_1 β-subunit amino acid sequence (Runswick & Walker, 1983) and the conserved region of the inhibitor protein reveals a region from residues 394–413 (Table I) which is very similar. This region of the β-subunit is highly conserved (Walker et al., 1985) and is composed of two clusters of charged amino acids, one acidic and one basic (Kagawa, 1984).

In order to investigate the role of the conserved region in the inhibitory activity of the inhibitor protein, peptides corresponding to amino acids 22–46 of the beef heart F_1I and amino acids 17–41 of the *S. cerevisiae* and the *C. utilis* F_1I were synthesized by the solid-phase method of Merrifield (1963). These synthetic peptides were then used to determine their effects on ATP hydrolysis catalyzed by the soluble beef heart F_1-ATPase and ATP synthesis catalyzed by beef heart submitochondrial particle bound F_1F_o-ATPase.

Additionally, the relationship of the conserved sequence of the inhibitor protein and the β-subunit and their role in inhibitor protein activity were explored. The homology between F_1I and the β-subunit along with the evidence that this region of the β-subunit is located within the binding site of F_1I led to the synthesis of three peptide analogs which correspond to β-394–413 and the two flanking regions, β-384–403 and β-404–423, respectively. These peptides were synthesized in an attempt to determine the size of the potential F_1I binding site. Additionally, two peptides which are perturbations of the β-394–413 sequence were also synthesized. The first perturbation, β-Gly, contained an insertion of five glycine residues between amino acids 403 and 404 of the β-394–413 sequence. The purpose of this insertion between the two charge clusters was to disrupt the α-helical secondary structure which is predicted by the calculations of Chou and Fasman (1978). The second perturbation was an attempt to use the same amino acids but with the two charged clusters in reversed order. This peptide, β-mirror, was synthesized so that the 404–413 region preceded the 394–403 region of the β subunit. This peptide should provide information on complementary charge requirements for successful binding and inhibition while maintaining α-helical secondary structure.

Kinetic analyses of these various peptides were performed at pH values ranging from 6.5 to 8.0 to determine whether

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Table I: Sequences of Synthetic Peptides Used in This Study

peptide[a]	sequence	M_r
A[b]	FGKREQAEEERYFRARAKEQLAALK	3025.6
B[b]	FVKRERATEDFFVRQREKEQLRHLK	3291.6
C[b]	FEKREKAQEDLYIRQHEKEQLEALK	3031.4
D[b]	DELSEEDKLTVSRAR KIQRF	2418.5
E	LQDIIAILGMDELSEEDKLT	2541.6
F	VSRARKIQRFLSQPFQVAEV	2361.6
G	DELSEEDKLTGGGGGVSRARKIQRF	2704.0
H	VSRARKIQRFDELSEEDKLT	2418.5

[a] A, bovine heart inhibitor protein 22–46; B, *S. cerevisiae* inhibitor protein 17–41; C, *C. utilis* inhibitor protein 17–41; D, bovine heart β subunit 394–413; E, bovine heart β-384–403; F, bovine heart β-404–423; G, bovine heart β-394–403-GGGGG-404–423 (β-Gly); H, bovine heart β-404–413–394–403 (β-mirror). [b] Sequences are shown to give maximal overlap of regions of similar amino acid sequence.

these peptides possess inhibitory characteristics that are similar to those of the natural F_1 inhibitor protein.

MATERIALS AND METHODS

Materials. The *t*-BOC amino acids and solvents utilized in the synthesis of the peptides were obtained from E. I. du Pont de Nemours and Co. Liquid hydrogen fluoride (HF) was obtained from Matheson Specialty Gas Co. HF was freshly distilled in an HF distillation apparatus obtained from Peninsula Labs. ATP, NADH, PEP, lactate dehydrogenase, pyruvate kinase, tricine, and PIPES were obtained from Sigma Chemical Co.

Biological Preparations. Soluble F_1-ATPase was prepared from beef heart mitochondria by the method of Knowles and Penefsky (1972), as modified by Gruys et al. (1985), including 1 mM PMSF in all buffers used prior to the ion-exchange step. F_1 was stored at 4 °C as a 2 M $(NH_4)_2SO_4$ precipitate in 0.25 M sucrose, 50 mM Tris-HCl, pH 8.0, 2 mM EDTA, and 4 mM ATP. Before use, F_1 preparations were desalted by centrifugal filtration using Bio-Rad P-6DG desalting gel and suspended in 20 mM PIPES, pH 6.5, containing 1 mM ATP and 0.1 M sucrose at room temperature.

Synthesis and Purification of Peptides. Peptides corresponding to beef heart F_1I 22–46, *S. cerevisiae* F_1I 17–41, *C. utilis* F_1I 17–41, and beef heart β-394–413, β-384–403, β-404–423, β-394–403-GGGGG-404–423 (β-Gly), and β-404–413–394–403 (β-mirror) were synthesized by solid-phase *t*-BOC chemistry in a Du Point Coupler 2200. At the each of each coupling cycle a sample of the peptidyl resin was removed and tested by the quantitative ninhydrin test (Sarin et al., 1981) to verify that a complete coupling had occurred. The peptides were then cleaved by reaction with liquid HF at 0 °C for 45 min in the presence of anisole (Stewart & Young, 1984) and then purified by reverse-phase HPLC using a Vydac C18 (2.2 × 25 cm) column on a Waters Model 600 chromatograph. The elution system consisted of buffer A, which is 0.1% TFA (v/v) in H_2O, and buffer B, which is 0.1% TFA in aqueous acetonitrile (90:9.9 CH_3CN:H_2O). The linear gradient was developed over 80 min, going from 0–80% buffer B. The elution of the peptide was monitored at 218 nm. In all cases the major peak corresponded to the desired product as determined by amino acid analysis. This component was collected and lyophilized.

Assay of Peptide Inhibitory Activity. The effects of the synthetic peptides corresponding to the beef heart F_1I 22–46, *C. utilis* F_1I 17–41, *S. cerevisiae* F_1I 17–41, and beef heart β-394–413 β-384–403, β-404–423, β-394–403-GGGGG-404–423 (β-Gly), and β-404–413–394–403 (β-mirror) were determined at several different pH values. The peptide and ATP concentrations were varied in the assay solution, which also contained 55 nM beef heart F_1, 0.4 mM NADH, 2.5 mM PEP, 2.0 mM KCl, 10 units of pyruvate kinase, and 10 units of lactate dehydrogenase. The buffer was either 20 mM PIPES (pH 6.5 and 7.0) or 20 mM Tricine (pH 7.5 and 8.0). This reduced the chance of high salt concentrations interfering with the association of the peptide with the F_1-ATPase complex. The total volume of the assay solution was maintained at 1 mL. The $MgCl_2$ concentration was kept at a 2.0 mM excess of the ATP concentrations in all the assays. The reactions were started by the addition of beef heart F_1 to the assay solution, and the rate of decrease of NADH absorbance was monitored at 340 nm. The cuvette temperature was maintained at 25 °C throughout the reaction by use of a circulating water bath. All of the reactions were determined in triplicate, and the average initial rate was used in the kinetic analysis.

Protein concentrations were determined by the method of Lowry et al. (1951), using BSA as the standard. The molecular weights of F_1 and the peptides beef heart F_1I 22–46, *C. utilis* F_1I 17–41, *S. cerevisiae* F_1I 17–41, and beef heart β-394–413, β-384–403, β-404–423, β-394–403-GGGGG-404–423 (β-Gly), and β-404–413–394–403 (β-mirror) were taken as 360 000 (Senior, 1979), 3025.6, 3031.4, 3291.6, 2418.5, 2541.6, 2361.6, 2704.0, and 2418.5, respectively.

Assay of Inhibition of ATP Regenerating System by Peptide Inhibitors. The use of an ATP regenerating system allows the rapid determination of any inhibitory activity for each of the peptides. However, it was necessary to determine whether these peptides were reacting specifically with the F_1-ATPase or if they had an inhibitory effect upon the enzymes of the ATP regenerating system. Therefore, each of the peptides was placed in a assay solution in which F_1-ATPase and ATP were replaced by the addition of 1 mM ADP. The peptide concentrations used were between 10 and 200 μM. These peptide concentrations had previously been found to produce a 50–75% reduction of the ATPase activity at pH 6.5. The lone exception to this was the β-mirror peptide, which had no effect upon the ATPase activity, and its concentration was 200 μM. The presence of any of these peptides in the ATP regenerating system resulted in a deviation of <1% from the control reaction which contained no peptide. The results indicate that the peptides were specifically affecting the ATPase activity of the F_1-ATPase complex and neither the lactate dehydrogenase nor the pyruvate kinase activity.

As an additional control against the nonspecific binding of any small peptide to the F_1-ATPase causing this inhibition, a peptide with the sequence corresponding to the C-terminus of beef heart F_1I 71–85 was synthesized and tested for inhibitory activity. This peptide resulted in no detectable inhibition at concentrations up to 1 mM peptide.

Assay of ATP Synthesis. Submitochondrial particles were prepared by the sonication of beef heart mitochondrial paste (Schuster et al., 1975). Frozen mitochondria were suspended in 0.25 M sucrose containing 2 mM EDTA and were converted to submitochondrial particles by sonication during two intervals of 2 min each. The mitochondrial were cooled on ice during the entire sonication to prevent overheating of the mitochondrial, which results in inactive enzyme. The resulting suspension was centrifuged for 90 min at 100000*g* at 3 °C to sediment the submitochondrial particles, which were then resuspended in 0.25 M sucrose containing 10 mM Tris-HCl,

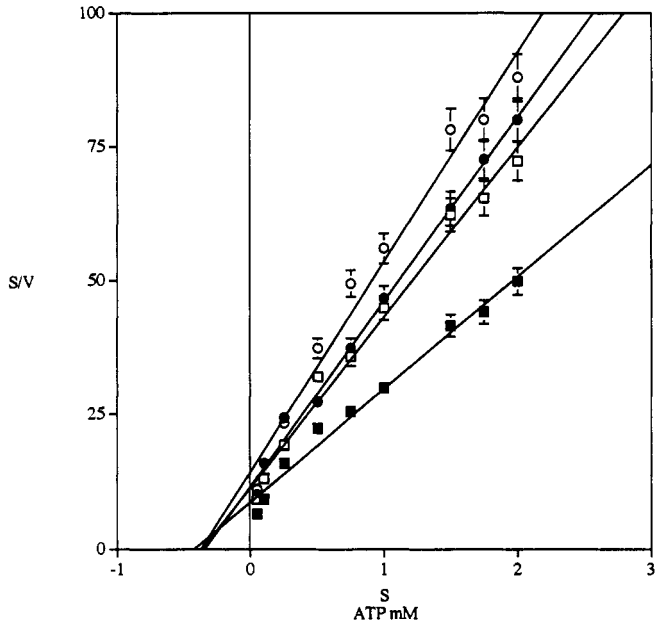

FIGURE 1: Hanes–Woolf plot of the inhibition of F_1-ATPase-catalyzed ATP hydrolysis at pH 6.5 with various concentrations of beef heart F_1I 21–46: (■) 0 μM, (□) 0.331 μM, (●) 0.662 μM, and (○) 0.993 μM. S/V is in min^{-1}, and [ATP] is in mM.

pH 7.5, at a protein concentration of 15 mg/mL. The submitochondrial particles were then frozen at −78 °C until used. The protein concentration was determined by a Biuret method (Layne, 1957).

The synthesis of [^{32}P]ATP from ADP and $^{32}P_i$ was measured in 1.0 mL of medium containing the following: PIPES, pH 7.0, 20 mM; sucrose, 200 mM; glucose, 50 mM; sodium succinate, 20 mM; rotenone, 3 μM; $MgCl_2$, 10 mM; hexokinase, 100 μg/mL; and ADP and P_i (with about 10^6 cpm/μmol of $^{32}P_i$) (Schuster et al., 1977). The $MgCl_2$ was kept 2.0 mM in excess of the ADP concentration in the reaction mixture. Peptide concentrations used in these reactions were in the range of 10–50 μM, which were sufficient to decrease the ATPase activity by more than 50% at pH 7.0 in the ATP hydrolysis assay.

Secondary Structure Predictions. The secondary structure predictions were made on the basis of the secondary structure parameters and the empirical rules of Chou and Fasman (1978) for the prediction of α and β regions.

RESULTS

Inhibition of F_1-ATPase by the Inhibitor Protein Peptides. An attempt to mimic the activity of the F_1 inhibitor protein led to the synthesis of three peptides which correspond to the conserved region of the beef heart, *C. utilis*, and *S. cerevisiae* inhibitor proteins. The effect of the beef heart F_1I 22–46 peptide on F_1-ATPase-catalyzed ATP hydrolysis can be seen in Figure 1. The Hanes–Woolf plot (Figure 1) shows a near intersection of the lines on the *x*-axis, which indicates either a noncompetitive-like inhibitor or a mixed inhibitor pattern. The I_{50} values over a pH range from 6.5 to 8.0 are presented in Table II along with their standard errors. A dose response–curve where the beef heart F_1I 22–46 inhibitor concentration is plotted versus V_{max} (Figure 2) shows that the efficacy of the inhibitor is reduced at basic pH (Figure 2B).

The *C. utilis* F_1I 17–41 peptide yielded similar results (Table II) with an I_{50} value of 10 ± 5 μM at pH 6.5, and the I_{50} value increases as the reaction medium becomes more basic.

The *S. cerevisiae* F_1I 17–41 peptide was found to have similar properties (Table II) to those of its intact inhibitor protein at pH 6.5–8.0. An I_{50} of 4 ± 3 μM was observed at pH 6.5, while the I_{50} has increased to 48 ± 19 μM at pH 8.0.

Table II: I_{50} Values for the Inhibitory Peptides at pH Values of 6.5, 7.0, 7.5, and 8.0

peptide	I_{50} (μM) at pH 6.5	7.0	7.5	8.0
beef F_1I 22–46	1.3 ± 0.4	1.1 ± 0.6	44 ± 21	24 ± 7
C. utilis F1I 17–41	10 ± 5	5 ± 3	43 ± 34	140 ± 70
S. cerevisiae F1I 17–41	4 ± 3	2.6 ± 2.3	17 ± 3	48 ± 19
beef β-394–413	20 ± 3	27 ± 24	61 ± 38	210 ± 130
beef β-384–403	41 ± 11	25 ± 15	118 ± 97	78 ± 32
beef β-404–423	21 ± 8	78 ± 31	97 ± 53	291 ± 160
beef β-mirror	>1000	>1000	>1000	>1000

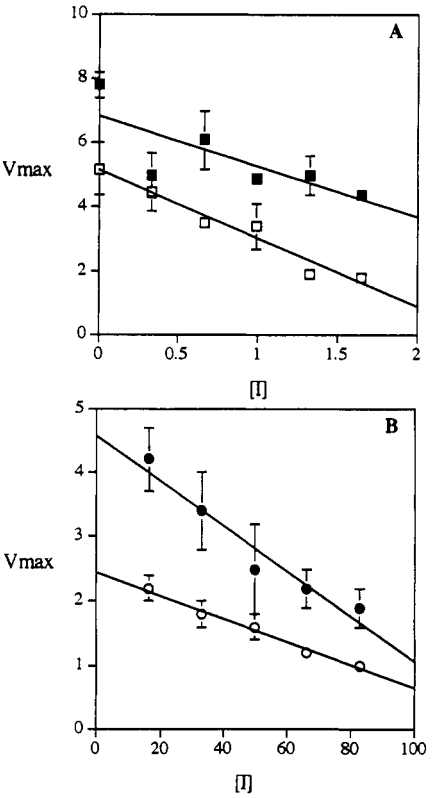

FIGURE 2: Dose–response plot of the inhibition of F_1-ATPase-catalyzed ATP hydrolysis (A) at pH 6.5 (■) and 7.0 (□) and (B) at pH 7.5 (●) and 8.0 (○) with various concentrations of beef heart F_1I 21–46; V_{max} is in 10^5 × min·mg/mol, and [I] is in μM.

Inhibition of F_1-ATPase by the β-Subunit Peptides. The kinetic effects of each of the five peptides, relative to those of the β-subunit 394–413 peptide, were determined at pH 6.5, 7.0, 7.5, and 8.0. The β-394–413 peptide, which is fairly homologous with the conserved region of the inhibitor protein (Table I), acts like a noncompetitive inhibitor at pH 6.5. The I_{50} value calculated for β-394–413 was 20 ± 3 μM at pH 6.5 (Table II). The I_{50} increases as the reaction medium is made more basic.

The β-384–403 peptide acts like a noncompetitive inhibitor of ATPase activity at pH 6.5. The I_{50} at pH 7.0 is 41 ± 11 μM (Table II), which is more than 2 orders of magnitude larger than the reported value for the intact beef heart F_1I (Klein et al., 1977); however, the I_{50} at pH 8.0 is still only 48 ± 32 μM.

When the β-394–413 peptide is extended toward the C-terminus, the peptide β-404–423 remains a potent inhibitor of ATPase activity. The I_{50} value is 21 ± 8 μM at pH 6.5 and 291 ± 160 μM at pH 8.0 (Table II). This type of inhibition is very similar to that obtained for the inhibitor protein and the peptides corresponding to the conserved region.

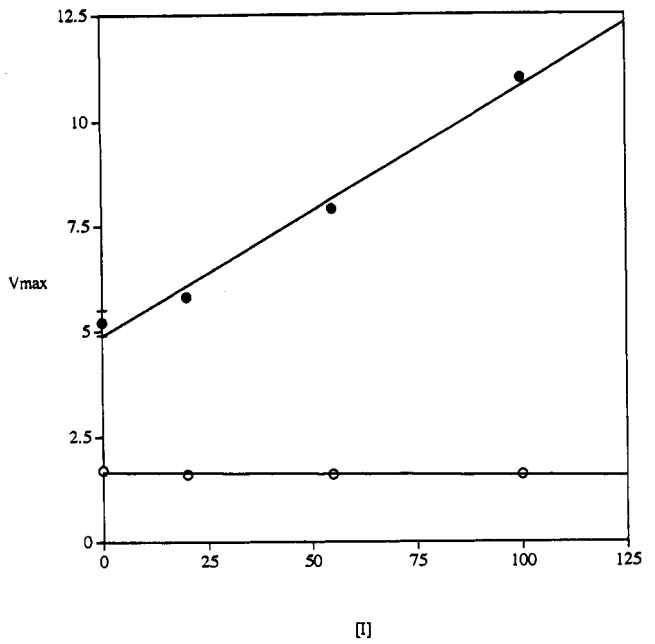

FIGURE 3: Dose–response plot of the activation of F_1-ATPase-catalyzed ATP hydrolysis at pH 6.5 (●) and 8.0 (○) with various concentrations of β-Gly; V_{max} is in 10^5 × min·mg/mol, and [I] is in μM.

The β-Gly peptide, which has five glycine residues inserted between the two clusters of charged side chains of β-394–413, was synthesized as a control peptide. However, this peptide is not inhibitory in nature but is instead an activator of ATPase activity catalyzed by the F_1-ATPase (Figure 3). The kinetic constants for pH values 6.5–7.5 show an approximate 50% increase in V_{max} and no change in K_m within experimental error. At pH 8.0, however, this activation is completely eliminated.

The β-mirror peptide contains all of the same amino acids and the same predicted secondary structure as β-394–413; however, the charges on the ends of the peptide have been reversed, which makes the N-terminus positively charged and the C-terminus negatively charged. The kinetic studies using this peptide at various pH values show the peptide to be a very weak inhibitor, with $I_{50} > 1000$ μM.

One significant difference between these peptides and the intact inhibitor proteins is that the truncated peptides do not appear to require a long incubation with Mg-ATP (Horstman & Racker, 1970) prior to initiation of the reaction in order for inhibition to occur. However, these peptides may still require ATP hydrolysis for inhibition prior to becoming active inhibitors, but they may require fewer catalytic turnovers for inhibition to occur due to their substantially smaller size.

Inhibition of F_1-ATP Synthetase by the Inhibitor Protein and β-Subunit Peptides. When the eight peptides were tested for their effect on steady-state ATP synthesis activity by use of $^{32}P_i$ incorporation into ATP (Schuster et al., 1977) catalyzed by submitochondrial particles, no inhibition was detected. However, other methods which allow for the simultaneous monitoring of ATP synthesis and hydrolysis (Schwerzmann & Pedersen, 1981; Beltran et al., 1986) might be able to detect some inhibition of ATP synthesis by these peptide analogs to the natural F_1-ATPase inhibitor protein.

Secondary Structure Predictions. The secondary structure predicted from calculations based on the method of Chou and Fasman (1978) is consistent with the beef heart F_1I 22–46 peptide being mostly an amphiphilic α-helix. However, the predicted secondary structure for the *C. utilis* F_1I 17–41 peptide shows a region of β-sheets that is predicted to occur at amino acids 26–28. The predicted secondary structure for the *S. cerevisiae* F_1I 17–41 peptide is entirely α-helical with no variation from this pattern. These predicted secondary structures closely match those observed for the entire beef F_1I (Frangione et al., 1981, Harris, 1984).

The secondary structure predicted for beef heart β-394–413 is almost entirely α-helical, as is the secondary structure of beef heart β-384–403. The N-terminus of the beef heart β-404–423 peptide is predicted to be α-helical, while the C-terminus is predicted to have a more random secondary structure. The β-Gly peptide is expected to have a segment of turn secondary structure separating the two clusters of α-helix on the two termini. In close agreement with the predicted secondary structure of the beef heart β-394–413 peptide, the secondary structure of the β-mirror peptide is expected to be entirely α-helical.

DISCUSSION

The inhibition of ATPase activity by synthetic peptides which correspond to the conserved region of the F_1I supports the proposal that the natural inhibitor proteins bind to the F_1 complex through an interaction involving the conserved region of the inhibitor protein. Previous studies which utilized proteolytic peptides derived from F_1I failed to detect inhibition by peptides which were smaller than 8000 Da (Dianoux et al., 1981, 1982). One possible explanation for this discrepancy may be the fact that the experimental conditions utilized only one inhibitor concentration and only one ATP concentration. The use of these conditions would have made the detection of a 10% inhibitory activity difficult to distinguish from the standard errors. In addition, the proteolytic enzymes were still present in these digested samples and could have interfered with any inhibitory activity.

The Hanes–Woolf plot (Figure 1) and the dose–response curve (Figure 2) show the same dependence on pH as the intact beef heart F_1I, which is most active in an acidic medium. The intact inhibitor protein also acts like a noncompetitive inhibitor of ATP hydrolysis (Horstmann & Racker, 1970). An I_{50} value of 1.1 ± 0.4 μM for the beef heart F_1I 22–46 was obtained at pH 6.5. This value is an order of magnitude larger than the $K_{i[app]}$ of 80 nM reported for the intact inhibitor protein (Klein et al., 1977). In addition, the I_{50} values obtained for the *C. utilis* 17–41 and *S. cerevisiae* 17–41 peptides were about 2 orders of magnitude larger than the reported $K_{i[app]}$ value of 10^{-7} M for the intact *C. utilis* and *S. cerevisiae* inhibitor proteins (Satre et al., 1975).

The fact that all three inhibitor peptides derived from the conserved region of F_1I showed a higher I_{50} than the $K_{i[App]}$ of intact inhibitor proteins may be due to a required size for a total disruption of the protein structure in the immediate vicinity of the inhibitor binding site. It would be interesting to prepare a synthetic peptide that had the same N-terminal sequences as these peptide analogs but had a C-terminal tail that was different from any of the intact inhibitor proteins. If this C-terminal tail were properly designed to form an α-helical structure, the full activity of the inhibitor protein might be restored. This would support the idea that the C-terminal tail is acting only as a spacer to distort the protein structure of F_1-ATPase.

The reliance of F_1-ATPase upon three cooperative catalytic sites (Gresser et al., 1982) and the protein sequence similarity between the 394–413 region of the β-subunit and the conserved region of the F_1I from beef heart (Runswick et al., 1986), *C. utilis* (Dianoux & Hoppe, 1987), and *S. cerevisiae* (Mat-

subara et al., 1983) led to our proposal that the inhibitor protein is active by mimicking one of the subunits of the F_1-ATPase complex and thereby disrupting inter- and/or intrasubunit interactions which are required for ATP hydrolysis. The assignment of this imitated region to the β-subunit is supported by the determination of the F_1I binding site during inhibition on the β-subunit of the F_1-ATPase (Klein et al., 1980, 1981; Jackson & Harris, 1988). A peptide fragment which was cross-linked to F_1I during inhibition is identical to the β-subunit β-394–495 (Jackson & Harris, 1988). The inclusion of the β-394–413 peptide in this F_1I binding site appears to support our proposed mechanism for ATPase inhibition by F_1I.

The ability of these peptides to inhibit ATPase activity in a manner characteristic of the intact inhibitor proteins may be useful in the determination of the molecular dynamics that are involved in the binding of F_1I to F_1. Both the conserved region of F_1I and the β-394–413 peptide are amphiphilic and α-helical in structure and contain two clusters of charged amino acids. These similarities in secondary structure and charge distribution seem to indicate that these features may be important in the binding of F_1I to the F_1-ATPase complex. It has been proposed that peptides which appear to be amphiphilic α-helical structures may be involved in binding to other proteins. One such case is a class of calmodulin binding peptides (DeGrado et al., 1985). This example is of particular interest, since calmodulin has been shown to bind beef heart F_1I in a Ca^{2+}-dependent reaction (Schwerzmann et al., 1985). This proposal is supported by various reports of aggregation of F_1I (Klein et al., 1981; Jackson & Harris, 1983) even under the denaturing conditions of SDS–PAGE (Klein et al., 1981). Similar aggregate formation was observed with model amphiphilic α-helical peptides (Kaiser & Kezdy, 1983).

The similarities of the peptides led to the synthesis of a series of peptides related to the β-394–413 peptide which were tested for inhibitory activity on the F_1-ATPase-catalyzed hydrolysis of ATP at various pH values. The results with the various peptides present an interesting array of kinetic properties.

The β-394–413 peptide has the greatest amount of homology with the F_1I conserved region and would be expected to be the best inhibitor of the β-subunit-derived peptides. This prediction closely matches the results obtained in kinetic analysis of this peptide; however, β-394–413 is not as good an inhibitor as beef heart F_1I 22–46. This difference in inhibitory activity may be related either to the spacing between the clusters of charged side chains or to the reduction in the number of cationic side chains present in the N-terminal portion of the beef heart β-peptide when compared to any of the peptides corresponding to the conserved region of F_1I.

The β-384–403 peptide remains a fairly potent inhibitor of ATPase activity, but differs from the peptides derived from F_1I in that its inhibition does not appear to be affected by the increase in pH which is a characteristic of these synthetic peptides as well as the intact F_1I (Horstman & Racker, 1970). This peptide is predicted to be entirely α-helical and has a cluster of anionic side chains at the C-terminus. This cluster of anionic side chains may allow this peptide to remain a potent inhibitor even at pH values greater than 7.0. One could speculate that the changes in the inhibitory activity of F_1I and the conserved peptides from F_1I may be due to the interactions of the cationic clusters with the β-subunit or other subunits in the vicinity.

The β-404–423 peptide is a good inhibitor of F_1-ATPase activity. The different kinetic results from β-404–423 and β-384–403 suggest that these two peptides may bind to different sites on the F_1-ATPase complex, and that the combination of the two peptides into a β-384–423 peptide may produce an inhibitor with a smaller K_i than either the β-384–403 or the β-404–423 peptide alone.

The most interesting peptide is the β-Gly peptide, which was originally designed to act as a control for inhibition. It was predicted that a series of glycine residues inserted between the two charge clusters would disrupt the α-helical secondary structure of the β-394–413 peptide and prevent any inhibitory activity. As predicted, this peptide does not have any inhibitory activity; however, it is a potent activator of ATPase activity. Similar activation of ATPase activity has been reported for bicarbonate, bisulfite, selenite, borate, chromate, and other anions as well as 2,4-dinitrophenol (Racker, 1962; Lambeth & Lardy, 1971; Pedersen et al., 1974; Ebel & Lardy, 1975; Moyle & Mitchell, 1975); however, this is the first report of a peptide having stimulatory activity. The activation of the ATPase activity by the β-Gly peptide could be explained by a change in the rate-limiting step, which is the release of the ADP from one of the three catalytic sites on the F_1-ATPase molecule.

The β-mirror peptide is an attempt to determine the spatial relationship of the charged clusters required for inhibition of the F_1-ATPase. The perturbation of the sequence by this method caused the loss of inhibitory activity at all pH values tested. The secondary structure of this peptide is predicted to be entirely α-helical just like the β-394–413 peptide. The great disparity of the inhibition constants for these two peptides suggests that the proper orientation of the α-helix and the charge clusters along this structure are required for binding of the peptide to the F_1-ATPase complex to cause inhibition. The charged clusters may be involved in an electrostatic interaction with some complementary charged site located on the F_1-ATPase molecule, and this complementary site could be located at a subunit–subunit interface.

The 394–413 region of the β-subunit has been suggested to be involved in the active site of the F_1-ATPase by Kagawa (1984) in his charge cluster hypothesis. To satisfy the stoichiometry of ATP synthesis ($3H^+$/ATP) (McCarty, 1979; Berry & Hinkle, 1983), more than three basic and three acidic groups are needed in one device. The acidic cluster comprises residues 394–400 of the beef heart β-subunit, and the basic cluster comprises residues 406–411 of the β-subunit. These clusters are highly conserved from bacteria, chloroplast, and mitochondrial F_1. These charge clusters are proposed to be located near the Rossmann fold, which may be involved in the binding of the nucleotide to the β-subunit (Kagawa, 1981; Hollemans et al., 1983). The binding of F_1I to this region of the β-subunit comprising residues 394–413 could greatly disrupt the interaction of these charge clusters and interfere with their role in the release and adsorption of protons through mutual electrostatic interactions. Further work with these peptides and the intact inhibitor protein is required to determine the number and position of their binding sites.

An alternative explanation of the inhibitory activity is that this region of the β-subunit is located at an interface between an α-subunit and the β-subunit. The presence of the inhibitor protein or one of the peptide analogs may be able to disrupt the contact between these two subunits by acting as a "wedge". This could cause the enzyme to be "frozen" in one conformation and would explain the need for only one inhibitor protein to inhibit F_1-ATPase activity. Intersubunit communications

between the α- and β-subunit have been observed (Ohta et al., 1980; Wise et al., 1981; Bragg et al., 1982; Issartel et al., 1983; Issartel & Vignais, 1984; Stan-Lotter & Bragg, 1986b) and can be disrupted by mutations and chemical modification, which resulted in a loss of catalytic activity (Stan-Lotter & Bragg, 1986a,c). Three types of conformational interactions have been proposed: from α to β, from β to α, and from β to β (Stan-Lotter & Bragg, 1986a,c). Either the α to β or the β to α interaction could provide an ideal location for the placement of a "wedge" peptide that mimics one of the subunits. From the results obtained with the synthetic peptide analogs to the β-subunit, it appears that the subunit that is mimicked by the inhibitor protein is the β-subunit. The two mechanisms of inhibition proposed above are not mutually exclusive, and the actual mechanism may involve both the charge clusters and the intersubunit interactions.

The wide variety of kinetic effects found for these peptides allow them to be useful probes of the molecular interactions that are involved in ATPase inhibition and activation. Since synthetic peptides are easily obtained in usable quantities, future experiments can use modified peptides designed specifically as probes in cross-linking experiments and to determine what affects increased mass and charge have on inhibitory activity.

The use of synthetic peptide analogs to the natural inhibitor protein opens many areas of future investigation. One possible use of these peptide analogs involves the preparation of inhibitor protein analogs which contain a photoactivated amino acid for site-specific cross-linking studies. A peptide of this nature would allow the determination of answers to several questions: To which subunit(s) do these peptides bind? Is it the same subunit(s) as the intact inhibitor protein? How many of these peptide analogs bind to the F_1-ATPase complex to cause inhibition? By moving these photoactivated amino acids along the length of the peptide, a picture of the binding environment can be determined as the label is moved form one side of the helix to the other (O'Neil et al., 1987). It will be interesting to determine whether these synthetic peptides bind to the β-subunit at the same location as the intact inhibitor protein.

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