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Sites of protein–protein interaction on the mitochondrial F_1-ATPase inhibitor protein

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1. We have investigated the structure of the mitochondrial F_1-ATPase inhibitor protein from ox heart by using a differential trace-labelling method. This method has also been used to determine sites on the inhibitor protein involved in binding to both the isolated mitochondrial ATPase (F_1) and to a specific anti-inhibitor antibody. 2. Native, free inhibitor was trace-labelled on its lysine and serine residues with [^{14}C]acetic anhydride, and inhibitor protein unfolded in guanidinium chloride or specifically bound to another protein, with [^{3}H]acetic anhydride. Exposure/concealment of residues was deduced from the $^{14}C/^3H$ ratios of the peptides in a proteolytic digest of the inhibitor, after separation by h.p.l.c. 3. None of the lysine or serine residues in the native inhibitor are as exposed as in the unfolded form. There is a gradient of reactivity, with residues 54–58 being most concealed and exposure increasing towards either end of the protein. A slight decrease in reactivity is noted in residues 1–3, suggesting that the *N*-terminus may be in a fairly restricted environment. These findings are discussed in the light of the predicted structure of the inhibitor protein. 4. All but one of the labelled residues increases in reactivity when inhibitor protein binds to F_1. The exception, Lys-24, is only slightly concealed. Hence, F_1 binding appears not to involve the lysine or serine residues directly. This finding is consistent with the view that the F_1–inhibitor interaction is hydrophobic in nature. 5. Complementary information was provided using an anti-inhibitor antibody that binds to a site on the inhibitor different from that at which F_1 binds. Binding of this antibody conceals residues 54, 58, and 65 considerably. This confirms that F_1 does not interact with these hydrophilic residues on the inhibitor protein.

INTRODUCTION

The mitochondrial ATP synthase (F_1-ATPase) is associated *in vivo* with a small peptide (M_r 10000) that inhibits both its ATPase and ATP-synthetic activities (Gomez-Puyou *et al.*, 1979; Harris *et al.*, 1979; Husain *et al.*, 1985). The association between the ATP synthase and this 'inhibitor protein' is modulated by the energy state of the mitochondrial membrane (van de Stadt *et al.*, 1974; Power *et al.*, 1983), suggesting that the inhibitor may play a regulatory role in mitochondria (Harris, 1984).

The inhibitor protein from ox heart has been shown, by using chemical cross-linkers, to bind to the catalytic (β) subunit of the F_1-ATPase (Klein *et al.*, 1980; Jackson & Harris, 1983). Since the amino acid sequences of both the inhibitor protein (Frangione *et al.*, 1981; Harris, 1984) and the β-subunit of F_1-ATPase from ox heart (Runswick & Walker, 1983) are known, this system is useful for the study of the interaction of an enzyme and its regulatory subunit at the molecular level.

One approach to this problem has been to modify different parts, or residues, of the inhibitor protein by specific reagents (proteinases, chemical reagents etc.), and to look for effects on inhibitory activity. These approaches have suggested that (*a*) most of the ten lysine residues are not essential for inhibitory activity (Klein *et al.*, 1980), (*b*) not all, but probably some (Harris, 1984) of the five histidine residues are involved, and (*c*) the first nine *N*-terminal residues are not essential for inhibitory activity, but the next 13 residues are required (Dianoux *et al.*, 1981).

The work described here uses an alternative approach. In the present case we modify the inhibitor protein, first bound to F_1 and secondly free in solution, by using a non-specific reagent (acetic anhydride) and, by the difference between the labelling patterns, determine the residues concealed when the inhibitor binds to F_1. This approach has the advantage that the inhibitor is actually in its binding site when modified, and thus in its correct alignment. Studies using modification of the free inhibitor (above) are inconclusive, because activity must be determined after modification, and it is often unclear whether the modification actually affects inhibitory activity or simply the kinetics or affinity of inhibitor binding.

Studies using non-specific reagents suffer from two drawbacks: (i) the highly modified protein may take up an abnormal conformation before labelling is complete, and/or (ii) the yield of modified and unmodified peptides may differ widely. Both these problems are overcome by using the dual-labelling technique described by Hitchcock-de Gregori (1982). Radioactive labelling of sample (associated) and control (free) protein is done separately with trace amounts of [^{3}H]- or [^{14}C]-acetic anhydride, so that, on average, less than one residue per protein molecule is labelled. This keeps conformational effects to a minimum. The two samples of radioactive protein are then mixed and completely acetylated, before digestion and separation, so that variations in yield are constant for

Abbreviation used: F_1, isolated soluble ATPase from mitochondria.

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any given peptide in the two proteins. The $^3H/^{14}C$ ratio in each peptide thus given the exposure of a peptide in the sample relative to the control protein.

This technique is complemented, in the case of the F_1–inhibitor interaction, by the availability of an antibody to the inhibitor protein which (*a*) binds to only a limited region of the protein (Husain *et al.*, 1985) and (*b*) does not affect the F_1–inhibitor interaction, that is, it binds to a region of the inhibitor protein that is not part of the F_1-binding site (Jackson & Harris, 1983; Husain *et al.*, 1985). Thus F_1 and this antibody must conceal different residues on the inhibitor protein. By using these reagents we are able to show that the F_1–inhibitor interaction does not involve the lysine residues of the *C*-terminal helical segment (residues 24–82) of the inhibitor, and is most probably an interaction with the hydrophobic side of the helical segment. This conclusion agrees with previous work on the thermodynamics of this interaction (Gomez-Fernandez & Harris, 1978). A preliminary report of this work has appeared elsewhere (Jackson & Harris, 1986).

MATERIALS AND METHODS

Mitochondrial F_1-ATPase and its inhibitor protein were obtained from ox heart as described by Power *et al.* (1983). The cross-linked inhibitor protein and the antibody to it were prepared as described by Husain *et al.* (1985). Specific anti-inhibitor antibody was separated from the IgG fraction by chromatography on inhibitor–Sepharose conjugate. The conjugate was prepared by CNBr activation of Sepharose 4B (Parikh *et al.*, 1974), followed by incubation of activated Sepharose (1 g) with 4 mg of cross-linked inhibitor protein. Specific anti-inhibitor IgG bound tightly to this column and was eluted by 0.1 M-glycine/HCl buffer, pH 2.3. The eluate fractions containing IgG were neutralized and used immediately. The capacity of such a column was greater than 0.5 mg of specific IgG/ml gel.

Differential trace labelling was performed as follows (see Hitchcock-de Gregori, 1982). A 1 mg (100 nmol) portion of inhibitor protein or 1 mg of inhibitor–protein complex in 1 ml of 50 mM-triethanolamine (adjusted to pH 7.5 with HCl) was incubated at 20 °C and five aliquots of 5 μl of 0.1 M-acetic anhydride (^3H- or ^{14}C-labelled, 25 μCi/μmol) in acetonitrile added at 5 min intervals. The samples were then incubated for a further 40 min and the reaction stopped with 10 μl of 1 M-Tris base. Hydrolysis of acetic anhydride in solution under these conditions limits acetylation to below 2 mol of acetyl groups/mol of inhibitor protein.

^3H- and ^{14}C-labelled samples were prepared in tandem (see below) and subsequently combined. In the case of the inhibitor–protein complexes, 0.75 mg of carrier inhibitor protein was added to the combined sample at this stage, the mixture heated at 100 °C for 2 min, then clarified by centrifugation. This served to partially purify the inhibitor protein (which is heat-stable) from the mixture.

The inhibitor protein was then adsorbed on to a Waters Sep-Pak C_{18} cartridge, previously equilibrated with water, and the cartridge washed with water and 10% (v/v) acetonitrile (5 ml each) to remove free label, buffers etc. The inhibitor protein was eluted with 30% acetonitrile and freeze-dried.

The dried protein was redissolved in 1 ml of 6 M-guanidinium chloride (adjusted to pH 9 with NaOH) and treated with 5 × 5 μl of pure, unlabelled acetic anhydride. The solution was maintained between pH 8.5 and 9.5 with 5 M-NaOH over a 1 h period. Guanidinium chloride was removed by exhaustive dialysis against 1% (v/v) acetic acid, followed by 50 mM-NH_4HCO_3. The completely acetylated protein was then digested with tosylphenylalanylchloromethane ('TPCK')-treated trypsin (10 μg of proteinase for 24 h at 20 °C), freeze-dried, and subjected to reverse-phase h.p.l.c. (see below).

Labelled peptides were collected, freeze-dried and, where indicated, digested with *Staphylococcus aureus* V8 proteinase in 2 ml of 50 mM-NH_4HCO_3 (5 μg of proteinase for 24 h at 20 °C), freeze-dried and re-chromatographed.

The yield of inhibitor protein during these steps was monitored by including a trace of ^{125}I-inhibitor protein (Power *et al.*, 1983) at the first stage. If pure inhibitor is used, about 40% of the starting material is present in the tryptic digest. With inhibitor complexed to a binding protein, only about 20% reaches the digestion stage, probably because some inhibitor is lost in the precipitate thrown down on heat treatment. Recovery from the Sep-Pak cartridge is about 60% of applied protein in both cases, but this step proved essential to remove free, labelled, acetate before chromatography. After digestion, the yields of the different peptides differed.

Peptides were separated by reverse-phase h.p.l.c. Mixtures were applied in 0.05% trifluoroacetic acid in

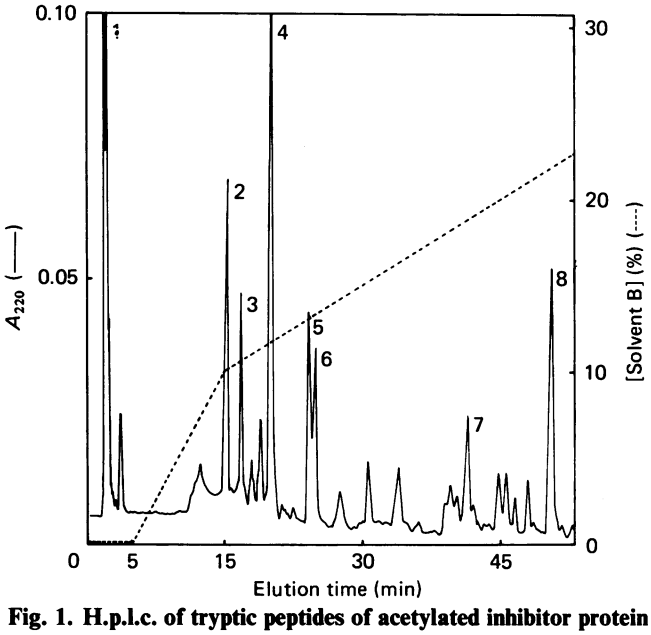

Fig. 1. H.p.l.c. of tryptic peptides of acetylated inhibitor protein

The inhibitor protein was completely acetylated, digested with tosylphenylalanylchloromethane-treated trypsin and chromatographed on a C_{18} reverse-phase radial compression column as described in the Materials and methods section. The gradient comprised three segments, as shown. Solvent A was 0.05% (v/v) trifluoracetic acid; solvent B was acetonitrile. A three-segment gradient proved necessary for optimal resolution of the nine peptides. For the resolution of the *S. aureus*-V8-proteinase subdigest (not shown), only two to four peptides were present; therefore a 5-min wash with solvent A followed by a gradient of 0–30% solvent B over 30 min was sufficient for good resolution.

Table 1. Peptides derived from acetylated inhibitor protein

Acetylated inhibitor protein was digested with tosylphenylalanylchloromethane-treated trypsin and subjected to reverse-phase h.p.l.c. as described in the Materials and methods section. The peptides were eluted in the order indicated by the numbers 1–8 (see Fig. 1). The peptides in peaks 2, 5/6, 7 and 8 were further digested with *S. aureus* V8 proteinase, and all peptides except those in peaks 1 and 4, which were unlabelled, were rechromatographed (see the legend to Fig. 1). 'V8' peptides are indicated by letters and elution times refer to the second h.p.l.c. step except those in parentheses. Abbreviation used: n.r., peptide not recovered. The one-letter notation for amino acids is used.

Peak	Elution time (min)	Peptide	Labelled residues
1	(1.75)	AR/EQAEEER	–
2a	14.5	GSE	S2
2b	n.r.	SGDNVR	S4
3	16.7	SSAGAVR	S10,S11
4	(21.0)	YFR	–
5a	13.0	IQR	–
5b	17.5	LQKE	K65
6	22.5	DAGGAFGKR	K24
7a	n.r.	DDD	–
7b	30.5	HKQSIKKLKQSE	K71-78, S73,S80
8a	n.r.	NE	–
8b	n.r.	IER	–
8c	n.r.	AKE	K39
8d	20.0	ISHHAKE	S54,K58
8e	27.5	QLAALKKHHE	K46,K47

water to a radially compressed C_{18} column (μBondapak; Waters) and eluted with a gradient of increasing acetonitrile concentration (see Fig. 1). The gradient was predetermined by using a du Pont Instruments series 8800 controller and pump, and applied at a flow rate of 2.5 ml/min. Peptides were detected by A_{220}, collected in 1.25 ml fractions, and freeze-dried.

^3H and ^{14}C were determined by using a Beckman LS7800 liquid-scintillation counter. Quench correction was accomplished by the 'H-number' method, using an γ-ray source incorporated into the instrument. A litre of scintillant contained 2,5-diphenyloxazole (4g), 1,4-bis-(5-phenyloxazol-2-yl)benzene (25 mg), Triton X-100 (250 ml) and toluene (750 ml). The peptide sequences were determined manually by the method of Chang *et al.* (1978).

Trifluoracetic acid (sequencer grade) and acetonitrile (h.p.l.c. grade) were obtained from Rathburn, Walkerburn, Peeblesshire, Scotland, U.K. Guanidinium chloride (Aristar), unlabelled acetic anhydride (Analar) and the scintillant chemicals were from BDH, Poole, Dorset, U.K. [^3H]- and [^{14}C]-acetic anhydride were supplied by Amersham International, Amersham, Bucks., U.K., and Sepharose 4B by Pharmacia Fine Chemicals, Uppsala, Sweden. Proteinases were from Sigma, Poole, Dorset, U.K.

RESULTS

Separation of acetylated peptides by reverse-phase h.p.l.c.

A protocol was developed for optimal separation of labelled, acetylated peptides from the inhibitor protein. The labelled inhibitor protein was freed from small molecules on a C_{18} Sep-Pak cartridge (see the Materials and methods section) and digested with trypsin. Since acetylation suppresses tryptic cleavage at lysine residues, this procedure yields nine peptides corresponding to cleavage at the eight arginine residues of the protein.

Reverse-phase h.p.l.c. of the digest separates five out of the nine peptides well, two poorly, and two not at all (Fig. 1). Only six out of these nine peptides were labelled, however (Fig. 1), and these, which contained lysine and/or serine residues, were collected for further treatment.

Peaks 2, 5/6, 7 and 8 were further digested with *S. aureus* V8 proteinase, which cleaves after glutamate residues. This subdigestion (*a*) separates the lysine and serine residues further, increasing the resolution of the method for locating concealed residues and (*b*) allows the separation of the peptides in peaks 5/6: one is cleaved, the other unaffected. Separation of these subdigests yielded a total of seven labelled peptides out of an expected nine, the last two being consistently difficult to recover from the column. The sequences of all the peptides produced, established by manual sequencing with 4-*NN*-dimethylaminoazobenzene-4′-isothiocyanate (Chang *et al.*, 1978), are shown in Table 1. The seven labelled peptides are seen to span virtually the entire length of the protein (Fig. 2).

Structure of the native inhibitor protein

The differential-trace-labelling method of Hitchcock-de Gregori (1982) was used to investigate the structure of the native inhibitor protein in free solution. The native inhibitor, in buffered solution at pH 7.5, was labelled (at < 2 mol of label/mol of inhibitor) with [^{14}C]acetic anhydride. The unfolded inhibitor, dissolved in 6 M-guanidinium chloride at the same pH, was labelled to similar extent with [^3H]acetic anhydride. The samples were then mixed, completely acetylated with unlabelled acetic anhydride, and the peptides prepared as described above. The activity of the labelled native inhibitor was not tested, but it has been shown that up to five lysine residues in the protein can be labelled without loss of activity (Klein *et al.*, 1980).

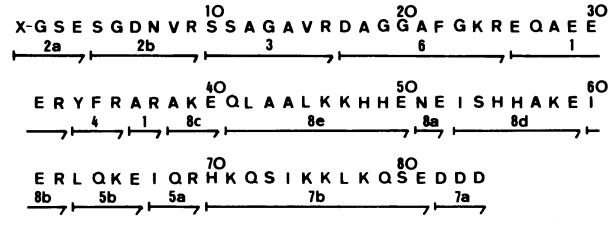

Fig. 2. Sequence of peptides derived from tryptic and *S. aureus*-V8-proteinase digestion of acetylated inhibitor protein

Sequences were determined manually with 4-*NN*-dimethylaminoazobenzene-4′-isothiocyanate ('DABITC') (Chang *et al.*, 1978). The numbers refer to the positions of the tryptic peptides during h.p.l.c. elution (Fig. 1), the letters to positions of elution after subdigestion with *S. aureus* V8 proteinase. The one-letter notation for amino acids is used. 'X' refers to the *N*-terminal blocking group, possibly formyl, according to Dianoux *et al.* (1984).

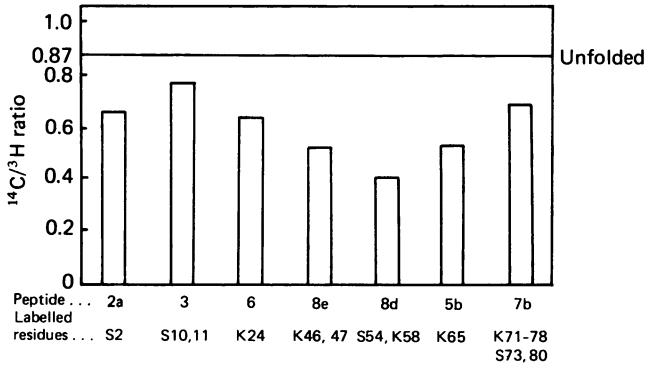

Fig. 3. Exposure of lysine and serine residues in the native inhibitor protein

Inhibitor protein, trace-labelled with [^{14}C]acetic anhydride in buffer at pH 7.5 and with [^{3}H]acetic anhydride in 6 M-guanidinium chloride at pH 7.5 was prepared, combined, and fully acetylated as described in the Materials and methods section, and the peptides separated as described in Fig. 1. The total d.p.m. recovered varied from 15×10^3 in peptides of low yield (e.g. nos. 3 and 8e) to 200×10^3 in peptides of high yield (e.g. nos. 5b and 6). In either case, the error on the observed $^{14}C/^{3}H$ ratio due to counting errors would be $< \pm 1\%$. The ratio of specific radioactivities of the $^{14}C/^{3}H$ acetic anhydride used was 0.87; thus a $^{14}C/^{3}H$ ratio of 0.87 indicates a residue completely exposed in the native inhibitor, with lower values indicating residues concealed in the native inhibitor.

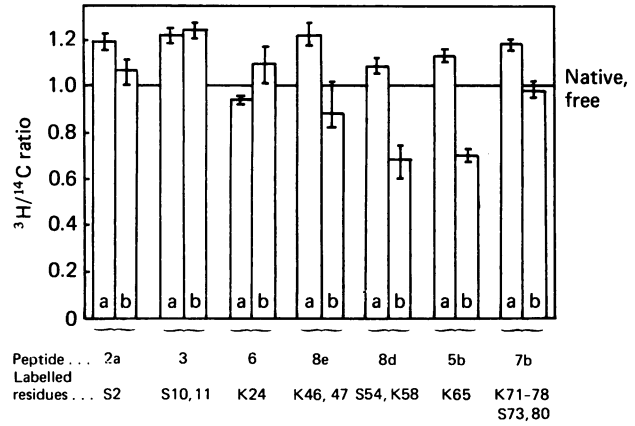

Fig. 4. Concealment of lysine and serine residues of inhibitor protein on binding F_1 or anti-inhibitor antibody

F_1–inhibitor complex was prepared by incubation of 10 μg of inhibitor protein with 1 mg (a 3-fold molar excess) of F_1 in 1 ml of 50 mM-triethanolamine/HCl, pH 6.5, containing 1 mM-MgATP at 37 °C for 10 min. The pH was adjusted to 7.5 with NaOH. A control sample of unbound inhibitor (10 μg) was prepared similarly, except that inactive F_1 was used, after cold denaturation at 4 °C for 16 h, and MgATP omitted. Inhibitor–antibody complex was prepared by incubation of 10 μg of inhibitor protein with 450 μg of affinity-purified antibody in 1 ml of 50 mM-triethanolamine/HCl, pH 7.5, at 37 °C for 60 min. A control sample was prepared similarly by using pre-immune IgG in place of anti-inhibitor. Peptides were prepared and separated as in Fig. 3. Since inhibitor–protein complex was labelled with [^{3}H]- and free inhibitor with [^{14}C]-acetic anhydride, the $^{3}H/^{14}C$ ratio gives the exposure of residues in the bound relative to the free inhibitor, a low value indicating concealment when inhibitor protein is bound to its complementary protein. The values are corrected here for the initial ratio of specific radioactivities (cf. above), so that a value of less than one indicates concealment of a residue on binding. Radioactivity found in the labelled inhibitor peptides was here much lower than in Fig. 3 (100–5000 d.p.m./peptide), since labelling of the complementary protein also occurs. The error bars shown represent the S.D. on the counting procedure, i.e. $\sqrt{n}$, where n is the total number of counts observed. 'a' and 'b' refer to inhibitor bound to F_1 and antibody respectively. The exposure of the residues in the inhibitor–protein complex relative to the totally unfolded inhibitor is obtained by multiplying the heights of the bars in Fig. 4 (bound/native) by those in Fig. 3 (native/unfolded). In all cases, the residues in the bound inhibitor are less exposed than in the unfolded inhibitor, as would be expected.

The results are shown graphically in Fig. 3. In view of the specific radioactivity of the ^{3}H- and ^{14}C-labelled samples of acetic anhydride, completely exposed residues should yield a $^{14}C/^{3}H$ ratio of 0.87 in this experiment. A value below this represents a peptide whose residues are concealed in the native protein.

Fig. 3 shows that, as expected, none of the lysine or serine residues of the native protein are as exposed as in the random-coil form. However, they also show a gradient of reactivity: those residues at either end of the protein appear to be relatively more reactive towards acetic anhydride than those around the centre, with a trough around residues 54–58. This is perhaps unexpected in view of the predicted helical structure for residues 24–80 in the native protein (Harris, 1984) and is discussed further below. We also note a slight decrease in reactivity at the very start of the protein (residues 1–3), suggesting that this terminal segment is in a more restricted environment in the native than in the unfolded form.

Interaction between the inhibitor protein and the isolated F_1-ATPase

Two complementary experiments were performed to investigate the site of inhibitor interaction with the ATPase. The inhibitor was initially complexed with a binding protein: in the first experiment with F_1, and in the second with a specific antibody that binds on the inhibitor at a site distinct from that at which F_1 binds (Husain *et al.*, 1985). The control in each case consisted of the inhibitor protein unbound, but with an equivalent amount of protein present: in the first experiment, cold-denatured F_1, and in the second, pre-immune IgG, neither of which bind to inhibitor protein. The bound inhibitor protein was labelled with trace amounts of [^{3}H]acetic anhydride and the free inhibitor with [^{14}C]acetic anhydride, as described above, in each case.

After purification of the inhibitor protein from these samples, the ^{3}H- and ^{14}C-labelled pairs were combined, acetylated and digested as above. The $^{3}H/^{14}C$ ratios of the various peptides are shown in Fig. 4. Since ^{3}H-labelling was done with bound inhibitor and ^{14}C with free inhibitor, the ratio (when corrected for the difference in ^{3}H and ^{14}C specific radioactivities) falls below 1 when a residue is concealed on binding.

Fig. 4 shows that all but one of the labelled residues of the native inhibitor protein increase in reactivity when this protein is bound to the ATPase. As expected, none of the residues becomes significantly more exposed than

in the unfolded inhibitor protein. The exception, Lys-24, is only slightly concealed, suggesting that F_1-inhibitor interaction does not directly involve lysine or serine residues. A lack of involvement of at least most of the inhibitor's lysine residues has also been inferred from activity studies with chemically modified inhibitor protein (Klein *et al.*, 1980).

Antibody binding has a distinctly different effect on the inhibitor protein, causing considerable concealment of residues 54, 58, and 65 (Fig. 4). In contrast, Lys-24, which was concealed by F_1 binding, is, if anything, exposed on antibody binding. First, this gives us confidence that this technique can detect specific effects of protein–protein interaction in the system we are studying. Second, since F_1 and the anti-inhibitor antibody bind independently to the inhibitor, neither affecting binding to the other, this finding confirms that F_1 cannot interact directly with residues 54, 58, and 65 or residues spatially close to them. Since these residues lie on one side of a helix (see below), the ATPase presumably binds to the opposite side.

It may seem odd that a polyclonal antibody can interact with only such a small region of this antigen, the inhibitor protein. Although this is difficult to explain, it is certainly a valid finding. We have shown previously that no precipitin line is obtained with this antibody and inhibitor protein in rocket immunoelectrophoresis (Husain *et al.*, 1985), suggesting that only one antigenic determinant is present, on average, per inhibitor molecule. This has been recently confirmed by immuno-electron microscopy (H. Tiedge, H. Lünsdorf & G. Schäfer, unpublished work).

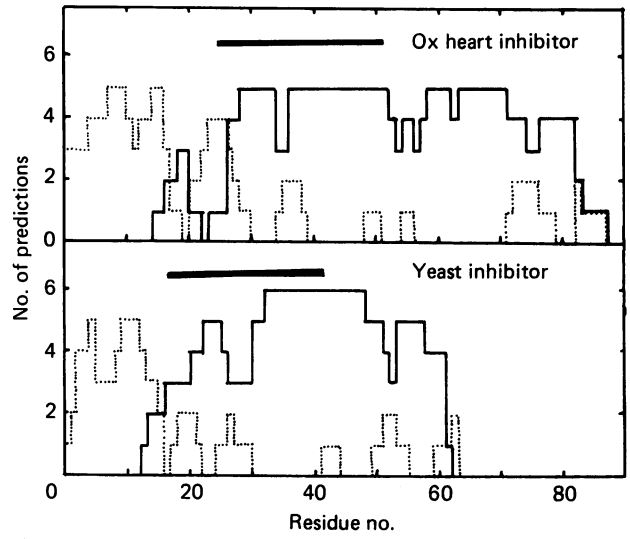

Fig. 5. Secondary-structure prediction for inhibitor protein

Prediction was made using a computerized method described by Eliopoulos *et al.* (1982). The vertical axis represents different probability weightings (6 being the most probable). ——, α-helix; · · ·, random coil. No β-sheet was predicted in any region of the protein. The upper diagram represents the predicted structure for ox heart inhibitor protein; the lower diagram *S. cerevisiae* inhibitor protein. The bar represents the region of homology between the two proteins.

DISCUSSION

Structure of the inhibitor protein

The inhibitor protein from mitochondrial resembles that better-known regulatory peptide calmodulin in being small, heat-stable and containing a large percentage of polar residues. Structural predictions (Fig. 5) also indicate that, like calmodulin, the inhibitor protein contains a high proportion of α-helix in its structure. This is confirmed by c.d., which shows that the protein has a molar ellipticity ($[\theta]_{222}$) of 25300 degrees·cm²·dmol⁻¹, indicating 70–80% helix (Harris, 1984). The lower value reported by Frangione *et al.* (1981) may have been due to problems in estimating protein concentration in solutions of this small protein.

In fact, a variety of predictive methods suggest the inhibitor protein comprises two distinct domains: an *N*-terminal region of random coil (residues 1–23) and an α-helical region covering virtually the rest of the protein (residues 24–82). This structure appears to be conserved during evolution: the yeast mitochondrial inhibitor (Matsubara *et al.*, 1983), with limited sequence homology, lacks a few residues at each end, but the two-domain structure is still present (Fig. 5).

Two further features of this structure are apparent. First, the helical region of the inhibitor protein is highly amphiphilic, having a high hydrophobic moment (Eisenberg *et al.*, 1982). A helical plot shows that all the charged groups of the helical section cluster on one side of the helix, whereas the hydrophobic groups form an 'oily patch' on the other side, as shown in Fig. 6. Calculation of the hydrophobic moment of these helices, by the method of Eisenberg *et al.* (1982), yields the high values typical of proteins that bind to surfaces. The hydrophobic moment of helix 60–77, for example, is 0.48, a value as high as any reported by these workers. Since no specific interaction between the inhibitor protein and the membrane has been observed (Power *et al.*, 1983), we suggest that the hydrophobic surface involved in inhibitor protein binding is on the β-subunit of F_1 (see below).

In addition, and probably as a result of this amphiphilicity, the inhibitor protein aggregates in solution around neutral pH. Aggregates have been shown by gel filtration (Klein *et al.*, 1982), n.m.r. (Harris, 1984), and even after gel electrophoresis in detergent (Jackson & Harris, 1983), suggesting that the aggregates formed are relatively stable. Aggregation is promoted by salt (P. J. Jackson & D. A. Harris, unpublished work), and thus probably involves alignment of hydrophobic patches on different inhibitor molecules, although the effects of organic solvents on aggregation suggest that hydrophobic bonding may not be the only factor involved.

In view of the probable helical conformation of the inhibitor protein, the inaccessibility of the lysine residues of the inhibitor protein to acetic anhydride was unexpected, particularly as Fig. 3 indicates a gradient of inaccessibility with the maximum in the middle of the helical domain (residues 54–58). On an α-helix in solution, all amino acid side chains should point outwards and thus be freely accessible from the solution. This paradox, however, may be explained by two additional effects: (i) salt-bridging between lysine residues and nearby negatively charged residues (glutamic acid, aspartic acid), which can be seen from the sequence (Fig. 2) to be nearby on the helix, and (ii) aggregation of the inhibitor protein in solution. The interactions will restrict reactivity of the residues within the helix either by

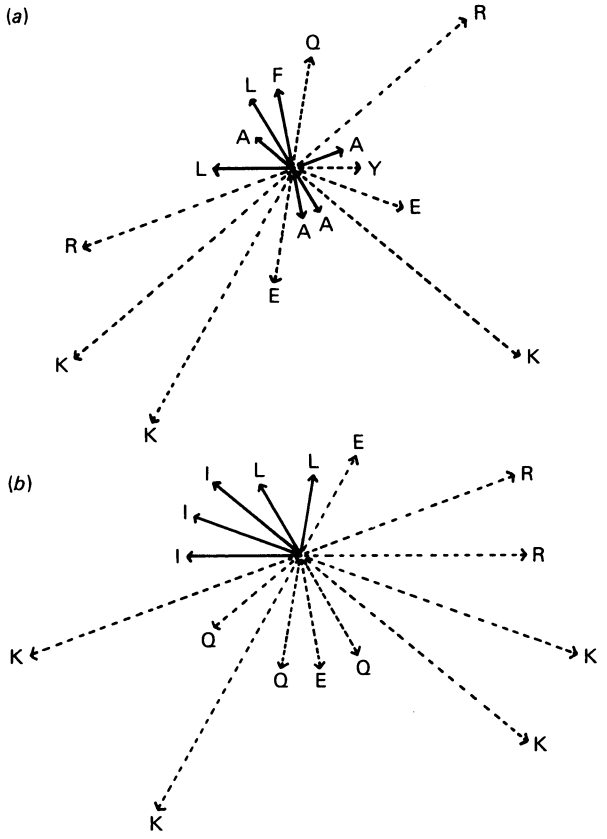

Fig. 6. Helical plots of residues in the helical segment of the inhibitor protein

The spacial arrangement of residues (*a*) 33–50 and (*b*) 60–77 is shown in helical plots (Eisenberg *et al.*, 1982). The hydrophobicity of a residue is represented by the length of its corresponding vector, positive hydrophobicities being shown by continuous lines and negative hydrophobicities by broken lines. Only residues that contribute significantly to the amphiphilicity are shown. The hydrophobic moments (Eisenberg *et al.*, 1982) of helical sections (*a*) and (*b*) are 0.24 and 0.48 respectively. For comparison, the hydrophobic moment of the amphiphilic helical segment of mellitin, a surface-binding protein, is 0.40 (Eisenberg *et al.*, 1982).

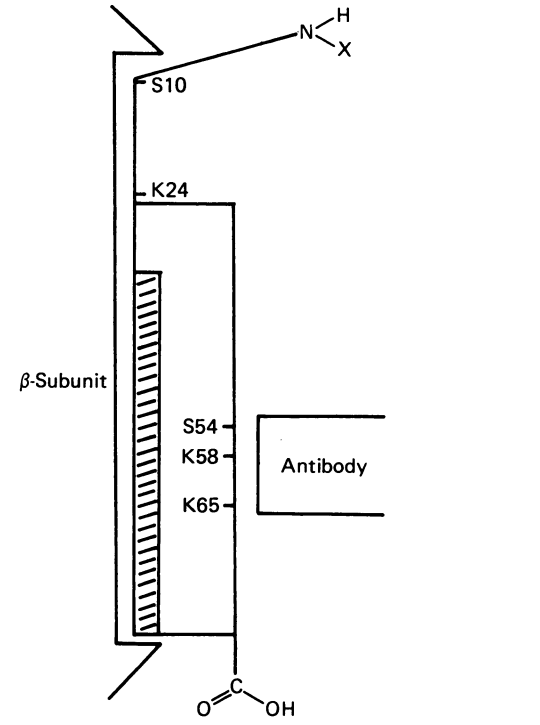

Fig. 7. Model for the interactions between inhibitor protein and F_1 antibody

This is described in the Discussion section. The hatched area represents the 'hydrophobic patch' on the inhibitor protein.

changing the pK_a of amino groups by interactions within the protein or by steric effects.

These effects may also explain the increase in reactivity of the inhibitor's lysine and serine residues on binding F_1 (Fig. 4). This is difficult to explain if the larger protein simply binds to the smaller, free, protein; but if in doing so it breaks down aggregates and/or neutralizes some of the negative charges in the salt bridges (lowering the pK_a of the amino groups involved), an increase in reactivity should be observed. In fact, F_1 must break down aggregates of inhibitor protein, since only 1 mol of inhibitor binds/mol of F_1 (Gomez-Fernandez & Harris, 1978) and it probably interacts little with the charged side of the helix (see below), so the aggregation effect is probably the major one.

Sites of interaction between the inhibitor and other proteins

Unexpectedly, no labelled residues on the inhibitor protein (with the possible exception of Lys-24) were concealed by F_1 (Fig. 4). Proteolytic removal of residues 1–9 does not affect inhibitor function (Dianoux *et al.*, 1981), so these residues might not be expected to show any change, but Fig. 4 indicates that lack of concealment is general to all residues studied. This might be explained if the inhibitor protein binds in a shallow hydrophobic groove on F_1 via the hydrophobic patch on its helical domain. This is in accordance with the finding that the F_1–inhibitor bond is stronger at higher temperatures, suggesting a hydrophobic interaction between the two (Gomez-Fernandez & Harris, 1978).

Studies using proteolytic digestion also implicate the *C*-terminal region of the random-coil section (residues 10–23) in the inhibition of F_1 by its inhibitor protein, and this is supported to some extent by the slight concealment of Lys-24 on the inhibitor by F_1. However, since this region is missing in the homologous *Saccharomyces cerevisiae* (baker's yeast) inhibitor (Matsubara *et al.*, 1983), it is uncertain how this region contributes to the function of the inhibitor protein.

The results obtained with a specific anti-inhibitor antibody confirm that F_1 cannot interact with the polar side of the helical domain of the inhibitor. The antibody clearly conceals (and thus presumably binds at or near) Lys-58 and -65, and Ser-54 (Fig. 4). Since the antibody and F_1 can both bind simultaneously to the inhibitor protein, as shown by functional (Husain *et al.*, 1985) and electron microscope studies (Tiedge *et al.*, 1986) F_1 clearly cannot bind in this region. [Our antibody can be contrasted with that of Dreyfus *et al.* (1981), which binds only to inhibitor when not bound to F_1, suggesting that it covers the F_1-binding site on the inhibitor protein.]

Thus F_1 appears to interact with its inhibitor protein

via a hydrophobic patch on one side of the helical domain of the inhibitor, with the possible additional involvement of the proximal region of the random-coil domain (residues 10–23). The requirement for F_1 to turn over before binding the inhibitor protein suggests that the complementary patch on F_1 may not be normally exposed to the solvent, appearing only transitorily during turnover (Power *et al.*, 1983). The antibody, on the other hand, binds to the polar face of the helix. These interactions are shown in Fig. 7.

The interaction between the inhibitor protein and F_1, via a hydrophobic face, thus contrasts with the interaction between cytochrome *c* (of similar size and charge) with cytochrome oxidase, which appears to involve complementary charge interactions (Ferguson-Miller *et al.*, 1978). Although confirmation of this conclusion awaits results from direct structural studies, it may be that this reflects the different roles of cytochrome *c*, as a substrate that must turn over, and inhibitor protein, as a regulatory element in mitochondrial ATP synthesis.

Since the present manuscript was originally submitted, Runswick *et al.* (1986) have shown that the *N*-terminus of the ox heart inhibitor protein is not blocked as stated above and by Dianoux *et al.* (1984), but 'frayed', with three species of inhibitor occurring, each with a different amino acid at its *N*-terminus.

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