Identification of the pH Sensor for Nucleotide Binding in the Uncoupling Protein from Brown Adipose Tissue†

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ABSTRACT: The transport inhibiting nucleotide binding to the uncoupling protein (UCP) has a unique pH dependence and has been postulated to be controlled by the dissociation state of a carboxyl group in UCP with p*K* 4.5 and, in addition only for the nucleoside triphosphate, by a group with p*K* 7.2. To prove this assumption and to identify the carboxyl group, Woodward reagent K (WRK) was applied to UCP. In mitochondria, WRK was found to inhibit binding of GTP in a noncompetitive manner using WRK in the millimolar range. In isolated UCP, GTP binding is inhibited by WRK at a 1 to 2 ratio to UCP, suggesting that WRK primarily reacts with only one carboxyl group. Prebound GTP protects against WRK reaction as monitored by GTP binding. The protection decreases from pH 5 to 7 due to better reactivity of WRK and less tight GTP binding. WRK does not inhibit H^+ transport by UCP but prevents GTP inhibition of H^+ transport. For elucidating the WRK target residue, the WRK derivatized group was labeled with [^{3}H] by reduction with [^{3}H]$NaBH_4$. Both GTP and GDP largely protected against WRK-dependent [^{3}H] labeling. CNBr fragmentation identified the region T121−M197 as the [^{3}H] incorporation site. Combined CNBr and tryptophane cleavage by the reagent 3-bromo-3-methyl-2-((2-nitrophenyl)thio)-3*H*-indole (BNPS) allowed to further delimit the 2.8 kDa peptide W173−M197 as the [^{3}H] label carrier which contains two acid residues E190 and D195. To further identify the residue, limited tryptic digestion in sarcosyl-treated UCP was performed, and a tryptic fragment enclosing E190 and D195 was isolated which carried most of the [^{3}H] label. Edman degradation showed the major [^{3}H] label at the eighth position corresponding to E190 and no peak at D195. Thus, the original postulate of the pH-sensing carboxyl group regulating both the nucleoside di- and triphosphate binding has been verified. It is identified as E190 situated in the fourth transmembrane helix. In total, now four residues close to the nucleotide binding sites in UCP have been determined.

The uncoupling protein (UCP)[1] of brown adipose tissue mitochondria seems at first sight to be about the simplest biomembrane carrier known so far. It transports the most elementary ion, H^+, down an electrochemical gradient, generated by the respiratory chain (Nicholls, 1979). In accordance with this apparent simplicity, UCP is a comparatively small protein of $M_r = 33$ kDa and a member of the mitochondrial carrier family (Aquila et al., 1985, 1987). Deviant from this simplicity, the transport activity of UCP is regulated by several factors. H^+ transport requires as obligatory cofactor free fatty acids (Locke et al., 1982a,b; Strieleman et al., 1985; Winkler & Klingenberg, 1994) and is inhibited by adenosine and guanosine di- and triphosphates (Nicholls, 1979).

The binding of these nucleotides has been instrumental for defining and isolating UCP from the mitochondria (Heaton et al., 1978; Lin & Klingenberg, 1980, 1982) . The binding site is located on the outer face of the inner membrane (Rafael & Heldt, 1978). The nucleotide binding occurs via two distinct states, a more loose binding state, where the binding does not yet cause inhibition of H^+ transport, and a tight binding state, where transport activity is inhibited (Huang & Klingenberg, 1995, 1996). The transition between these two states is relatively slow. Binding of some fluorescent nucleotide derivatives even arrests UCP in the uninhibited state (Klingenberg, 1984; Huang & Klingenberg, 1995, 1996). Distinct conformational changes could be shown to be associated with this transition between the two binding states (Huang & Klingenberg, 1996).

A third regulatory factor overlapping the influence of free fatty acids and of purine nucleotides is the pH. The striking influence of the pH on the affinity of nucleotides should regulate the uncoupling activity in the presence of nucleotide (Lin & Klingenberg, 1982; Klingenberg, 1988). Basically, nucleotide binding affinity decreases with the pH. The profile of this pH control, as shown in pK_D versus pH plots, is characterized by one break for nucleoside diphosphate or two breaks for the triphosphate (Klingenberg, 1988; Huang & Klingenberg, 1995). From a detailed investigation of the pH dependence, we concluded that the H^+ dissociation at a carboxyl group near the binding site for the phosphate moiety is a major factor in the pH control. Whereas this group is involved in the binding of both nucleoside di- and triphosphates, another H^+-dissociating group with a higher p*K* controls also the nucleoside triphosphate binding and causes a precipitous fall of the affinity above pH 7.2.

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[1] Abbreviations: BNPS, 3-bromo-3-methyl-2-((2-nitrophenyl)thio)-3*H*-indole; CNBr, cyanogen bromide; CB, cyanogen bromide fragment; SDS−PAGE, sodium dodecyl sulfate−polyacrylamide gel electrophoresis; UCP, uncoupling protein; WRK, Woodward reagent K.

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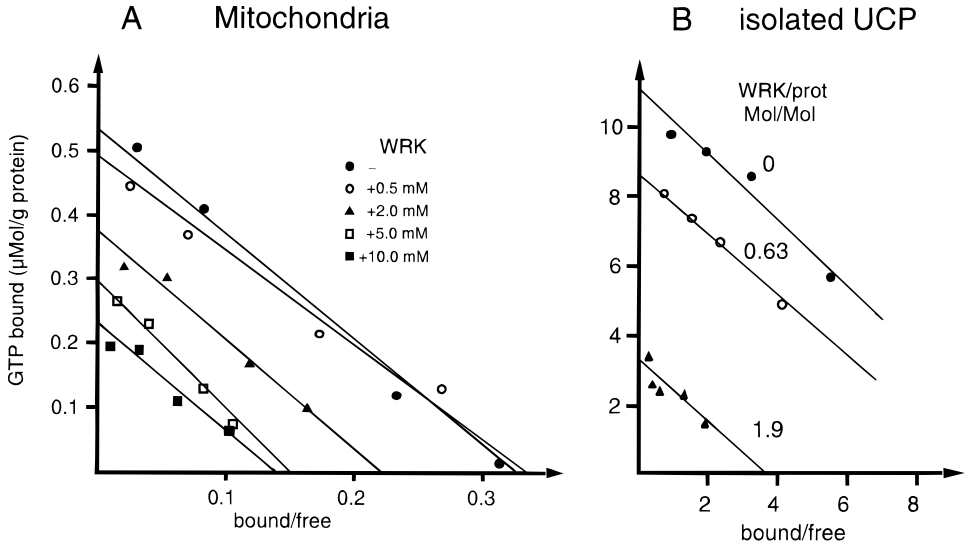

FIGURE 1: Influence of WRK on [^{14}C]GTP binding to mitochondria and to isolated UCP. (A) Mass action plot of [^{14}C]GTP binding to mitochondria. For the binding of GTP, mitochondria were incubated at 7 mg of protein/mL in 5 mM MES, pH 6.0, 1 mM EDTA, 300 mM sucrose. After addition of increasing concentrations of WRK (0−10 mM) to aliquots of the mitochondria suspension and stirring for 5 min at room temperature, the samples were centrifuged and the mitochondria were resuspended at 8 mg of protein/mL with 10 mM MES, 10 mM Pipes, pH 6.0, 1 mM EDTA, 300 mM sucrose. Portions of 4 × 100 μL of each pretreated mitochondria suspension were incubated with increasing concentrations of [^{14}C]GTP with a specific activity of 1.1 × 10^{13} dpm/mol for 1 h at room temperature. After centrifugation the pellet was dissolved in 100 μL of 2% SDS for scintillation counting. Control values for unspecific [^{14}C]GTP absorption were obtained by preincubating WRK untreated mitochondria with 500 μM unlabeled GTP prior to addition of [^{14}C]GTP. These values were subtracted from the total binding to yield the UCP specific binding. (B) Influence of WRK on GTP binding to isolated UCP. Mass action plot of GTP binding. Isolated UCP (0.24 mg of protein/mL) was incubated with WRK in 5 mM MOPS, pH 6.8, for 20 min at 10 °C at (▲) WRK:UCP = 1.9 and (○) 0.63 (mol/mol) and (●) without WRK. Four aliquots (50 μL) from each sample were now incubated with increasing concentrations (2−14 μM) of [^{14}C]GTP with a specific activity of 7 × 10^{12} dpm/mol for 30 min at 0 °C. [^{14}C]GTP binding was determined by the "anion exchange method" as described by Klingenberg et al. (1986). The samples were passed through a small column containing 30 mg of wet Dowex 1 × 8 (Cl^- form), and the bound [^{14}C]GTP was determined by scintillation counting of the eluate. The data were evaluated in a mass action plot.

In view of our conclusion that at least one carboxyl group is critically controlling the nucleotide binding, we employed here the carboxyl group modifying Woodward reagent K (WRK). We will show that WRK already at low concentrations causes a highly specific inhibition of the nucleotide binding and thus relieves H^+ transport. The specific protection against WRK by nucleotides identifies one carboxyl group involved in the nucleotide binding, in agreement with our previous postulate. We further identified this regulatory and WRK-sensitive carboxyl group as E190 located in the fourth transmembrane helix near the cytosolic site.

MATERIALS AND METHODS

Chemicals. Hydroxyapatite was prepared as described in Bernardi (1971). The detergent *n*-decylpentaoxyethylene ($C_{10}E_5$), the tryptophan reagent 3-bromo-3-methyl-2-((2-nitrophenyl)thio)-3*H*-indole (BNPS), and Dowex 1 × 8 (200−400 mesh) were purchased from Fluka (Ulm). Woodward reagent K (WRK) and *N*-lauroylsarcosine were from Sigma (Munich). [^{3}H]$NaBH_4$, [^{14}C]GTP, Hyperfilm-MP, and Amplify were obtained from Amersham, and trypsin (TPCK) was from Merck (Darmstadt). Egg yolk phospholipid was isolated from fresh eggs as described by Wells and Hanahan (1969) and purified with Alumina B Super I from ICN Biomedicals as described previously (Winkler & Klingenberg, 1994).

Isolation of UCP. Brown-fat mitochondria from cold-adapted hamsters were prepared as described previously (Lin & Klingenberg, 1982). UCP used for binding experiments and radioactive labeling was isolated with Triton X-100 according to the procedure of Lin and Klingenberg (1982), without the sucrose gradient centrifugation step. For the preparation of proteoliposomes, UCP was isolated with $C_{10}E_5$ as described in Winkler and Klingenberg (1992, 1994). [^{14}C]-GTP binding to mitochondria and to isolated UCP was performed as described in detail in the legend to Figure 1.

Reconstitution of UCP. Egg yolk phospholipids (30 mg/mL) were sonicated in the presence of 150 mM sodium phosphate, pH 6.2, 0.3 mM EDTA for 10 min. After stepwise addition of the detergent ($C_{10}E_5$) at 0 °C to a final detergent/phospholipid ratio of about 1.3 (w/w), isolated UCP was added to a phospholipid/protein ratio of 110 (w/w). The mixture was then diluted to a final concentration of 19 mg of phospholipid/mL and 0.17 mg of protein/mL. Vesicle formation by removal of detergent with Amberlite XAD-2, exchange of external solutes, and H^+ transport measurements were carried out as described by Winkler and Klingenberg (1994).

Reaction of Woodward Reagent K (WRK) with UCP and [^{3}H] Incorporation with [^{3}H]BH_4. Triton X-100 solubilized UCP (0.26 mg/mL) was incubated with 14 μM WRK in 5 mM Mops, pH 6.8, for 10−20 min at 0 °C. The mole ratio of WRK to UCP was 1.9:1. As a control, UCP was first incubated with 100 μM GTP for 20 min at 10 °C prior to reaction with WRK under the same conditions**.** To 0.45 mL of reacted UCP five portions of 1 μL of 0.2 N ice-cold solution of [^{3}H]$NaBH_4$ (specific activity 2.5 mCi/μmol) in 10 mM NaOH were added in 2 min intervals. The final molar ratio of [^{3}H]$NaBH_4$ to WRK was 150:1. For cleavage with CNBr and gel electrophoresis, the protein was precipitated with trichloroacetic acid in 34% acetone and 4.5% trichloroacetic acid. After 1 h, the protein was obtained by

centrifugation and two washings with 250 μL of 50% aqueous acetone and once with 250 μL of methanol. The protein yield was usually 50−60%.

CNBr and BNPS Cleavage. The protein pellet was dissolved in 80% formic acid at a final concentration of 6 mg/mL with a 500-fold CNBr molar ratio to the methionine of UCP. The cleavage reaction was carried out under N_2 for 16 h at room temperature. The reaction was stopped by dilution with water (1:10), and the sample was freeze-dried. For cleavage with CNBr and BNPS (Fontana, 1972), the protein was likewise dissolved in 80% formic acid, but BNPS (100 mol/mol of protein) and CNBr were added simultaneously.

Digestion of Labeled UCP with Trypsin. The precipitated protein (300 μg) was suspended in 12.5 μL of 4% *N*-lauroylsarcosine in 200 mM NH_4HCO_3, pH 9.1, and partially dissolved by shaking for 15 min at 37 °C . To avoid cross-linking of SH groups, 10 μL of 100 mM *N*-ethylmaleimide was added. This was followed by dilution with water to a final protein concentration of 1.2 mg/mL. Trypsin was then added to trypsin:protein 1:20 (w/w). After incubation for 90 min at 37 °C, a second portion of trypsin was added to a final trypsin:protein ratio of 1:10 (w/w), followed by a further 90 min digestion. The protein dissolved only poorly in *N*-lauroylsarcosine, but after the two-step cleavage with trypsin the solution became clear. The supernatant contained about 90% of [^{3}H]-labeled peptide. After freeze-drying, the lyophilizate was washed once with 100 μL of methanol plus 300 μL of acetone and with 50 μL of methanol plus 100 μL of acetone to remove the detergent. About 85% of [^{3}H]-labeled peptides were recovered.

Gel Electrophoresis for Analyzing Peptides. For separation of CNBr fragments and tryptophanyl peptides, the gel system for small peptides by Schägger and von Jagow (1987) was used. The lyophilized samples were dissolved in the SDS solution consisting of 4% SDS, 12% glycerol, 50 mM Tris, pH 6.8, and 2% 2-mercaptoethanol. After electrophoresis, gels were stained with Coomassie blue.

Purification of the Tryptic Fragment with GTP-Sensitive WRK Incorporation for Edman Degradation. (A) The fragments were separated on SDS−PAGE (Figure 7) that was cut into 1-mm-thick slices. The peptides were eluted from the slices by sonication with 0.1% SDS/10 mM NH_4HCO_3 and shaking overnight. The eluates were assayed for [^{3}H] radioactivity by liquid scintillation counting and freeze-dried. The detergent and glycerol were removed by washing with a mixture of 1 volume of methanol and 3 volumes of acetone and then with 1 volume of H_2O and 9 volumes of acetone. The dried peptides were dissolved in 100% trifluoracetic acid and applied for Edman degradation.

(B) The resulting tryptic fragments were first dissolved in 0.2% SDS, and after addition of trifluoroacetic acid to 0.1%, 70% of [^{3}H]-labeled peptides were found in the supernatant. The supernatant was applied on a reversed-phase HPLC RP-8 column, which had been equilibrated with 0.1% trifluoroacetic acid. Peptides were eluted with a gradient of 0−80% acetonitrile in 0.1% trifluoroacetic acid. The main [^{3}H]-containing peak fraction, eluated at about 50% acetonitrile, was dried and analyzed by Edman degradation.

RESULTS

In a first exploration, we studied the influence of WRK on the nucleotide binding to mitochondria. GTP was the preferred nucleotide for these studies since it does not bind to the ADP/ATP carrier. Further, GTP binding was found to be somewhat stronger than the often used GDP. For this purpose, mitochondria were first incubated with WRK, and excess WRK was removed by washing prior to the binding assay with [^{14}C]GTP. The results in Figure 1A show that WRK inhibits [^{14}C]GTP binding in an uncompetitive manner. This would be expected for covalent incorporation of WRK to a carboxyl group involved in the nucleotide binding.

All further experiments concentrated on the isolated UCP. Here we found to our surprise that extremely low concentrations of WRK were sufficient for complete inhibition (Figure 1B). Already at a molar ratio of 1.9 WRK/UCP monomer, 80% of the GTP binding sites are blocked by WRK. This is illustrated in Figure 1B in a mass action plot. Also in the solubilized UCP after treatment with WRK the affinity remains unchanged; however the binding is decreased in an uncompetitive manner.

Although this highly sensitive response of the nucleotide binding to WRK strongly supports the contention of the involvement of a carboxyl group (glutamate or aspartate) in the nucleotide binding, the possibility remains that this group only indirectly affects nucleotide binding and is not at the binding site as originally postulated (Klingenberg, 1988). A protection against the modification of a carboxyl group by nucleotides would indicate direct involvement of the carboxyl group. This, in fact, is shown in Figure 2. In this experiment the influence of three parameters on the nucleotide binding are combined, the dependence on the amount of WRK, the dependence on the pH, and the protection of nucleotides against modification by WRK. A dependence on the pH was important in view of its strong influence on the nucleotide binding affinity and also a possible influence of the pH on the WRK reactivity. For good protection GTP is added at near saturating concentrations, and therefore only a low pH influence on the GTP binding is seen. Preincubation with GTP protects, to a large extent, against the suppression of GTP binding by WRK. This protection decreases by increasing the pH, in line with the strong decrease of the affinity of GTP. Correspondingly, also with a higher pH much less WRK is required for the inhibition of nucleotide binding. For example, at pH 6.5 and 7 and at a molar ratio of WRK to UCP of only 2, the binding is inhibited by about 75%. In conclusion, these data strongly support the contention that the WRK-sensitive carboxyl group is directly involved in the nucleotide binding. The only small molar excess of WRK to UCP (WRK/UCP < 2) required to inhibit largely the nucleotide binding suggests that there is only one WRK-sensitive carboxyl group involved in the nucleotide binding.

Of great interest is the effect of WRK on the H^+ transport activity of UCP. To this purpose, UCP was reconstituted into proteoliposomes. As shown in Figure 3, lauric acid is added as an activator for the H^+ transport above the spontaneous activity due to residual endogenous fatty acids. WRK does not inhibit the H^+ transport. A preincubation with WRK precludes inhibition by GTP, whereas in the untreated liposomes GTP inhibits by about 70%. Thus, clearly WRK is not an inhibitor of the H^+ transport activity by UCP.

Identification of Incorporation Site. Since we have shown that WRK interacts with the GTP-binding site at nearly equimolar amounts, the preconditions for identifying the

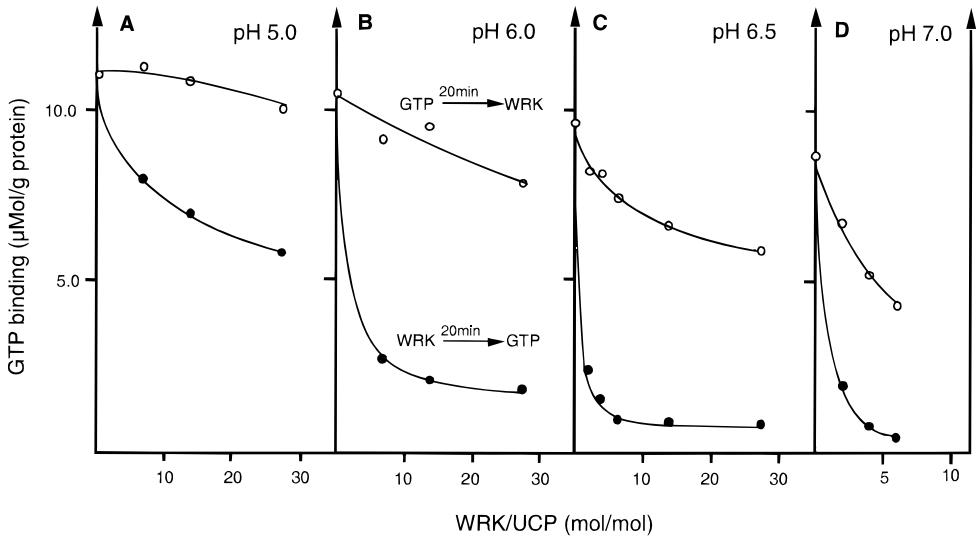

FIGURE 2: Influence of WRK on GTP binding to isolated UCP. Dependence on pH. UCP isolated with TX-100 was incubated in 5 mM MES at (A) pH 5.0, (B) pH 6.0, and (C) pH 6.5 and in 5 mM Pipes at (D) pH 7.0 at a final concentration of 0.25 mg of protein/mL. (●) Inhibition of GTP binding by preincubation with WRK. Aliquots of 125 μg in 50 μL of protein were incubated for 20 min at 10 °C with increasing concentrations of WRK at mole ratios of WRK:UCP = 0 to 27. For binding measurement [^{14}C]GTP (final concentration 18 μM) was added, and after incubation for 20 min at 10 °C, 50 μL of each sample was applied to a column containing 30 mg of Dowex as described by Klingenberg et al. (1986). (○) Protection by GTP against WRK derivatization. [^{14}C]GTP was first incubated with aliquots of protein (125 μg in 50 μL) in a final concentration of 18 μM WRK for 20 min at 10 °C. Then increasing concentrations of WRK were added, and after a further 40 min incubation the samples were assayed for [^{14}C]GTP binding.

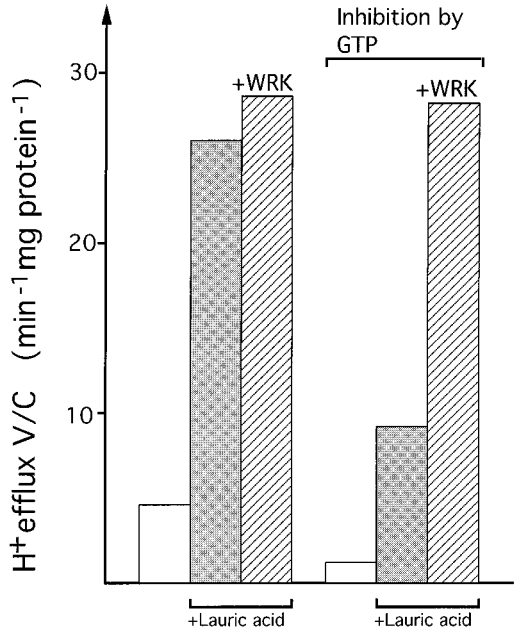

FIGURE 3: Effect of WRK on H^+ transport and the inhibition by GTP. For H^+ efflux measurements, proteoliposomes were prepared as described under Materials and Methods and loaded with 100 mM Na^+ phosphate, pH 6.2. After vesicle formation the external solutes were exchanged with an isoosmolar buffer containing 210 mM sucrose, 0.5 mM Hepes, 0.5 mM Pipes, 0.2 mM EDTA, pH 6.9, by passage through a Sephadex G-75 column. For WRK pretreatment, 1 mM reagent was added to an aliquot of the vesicles and incubated for 5 min. H^+ transport was measured after 5-fold dilution with buffer (described above) containing additionally 38 mM K^+ gluconate. H^+ transport was activated with 250 μM lauric acid and inhibited by preincubation with 100 μM GTP for 2 min. The H^+ efflux rate was measured at 10 °C on addition of 2 μM valinomycin. The H^+ efflux capacity was determined by addition of 1 μM CCCP.

residue which reacts with WRK are very favorable. Moreover, since the interaction of WRK with UCP can be inhibited by GTP, a clear blank value reflecting the incorporation to possible other residues is possible. The reaction of WRK with the carboxyl group results in various products according to the pH and further treatments. To identify the incorporation, we radiolabeled the adduct of the carboxyl group and WRK with [^{3}H]$NaBH_4$ according to the pioneering work of Jennings on band 3 protein (Jennings & Anderson, 1987). This reduction leads to cleavage of the adduct and formation of the alcohol from the carboxyl group which gives a stable [^{3}H] incorporation.

$$RCO_2{-}WRK + [^3H]NaBH_4 \rightarrow RC[^3H]_2OH + WRK'$$

A typical experiment for the derivatization by WRK of UCP and the subsequent incorporation of [^{3}H] is shown in Figure 4. UCP is exposed to WRK plus [^{3}H]$NaBH_4$ in the absence and presence of GTP in order to differentiate the [^{3}H] incorporation into the binding site from unspecific labeling. The radioautogram shows a clear decrease of [^{3}H] incorporation into UCP in the presence of GTP.

For a first assignment of the incorporation, we used CNBr cleavage. Here we could greatly profit from our previous work on the 2-azido and 8-azido ATP incorporation to UCP (Winkler & Klingenberg, 1992; Mayinger & Klingenberg, 1992). The nomenclature for the CNBr fragment starts from the end terminal and is based on the previous identifications (Aquila et al., 1985; Winkler & Klingenberg, 1992). A gel specially designed for the resolution of small fragments was used (Schägger & Von Jagow, 1987). In general, the CNBr cleavage of UCP is difficult for not yet fully understood reasons and requires careful preparation of the denatured protein to expose the cleavage sites as much as possible (Winkler & Klingenberg, 1992). In particular, the second cleavage site with methionine adjacent to threonine is known to be relatively resistant to CNBr (Waxdal et al., 1968) as evident from Figure 4 where CB2+3 form the major band. The distribution of [^{3}H] in these peptides is shown in a radioautogram which clearly shows the drastic suppression

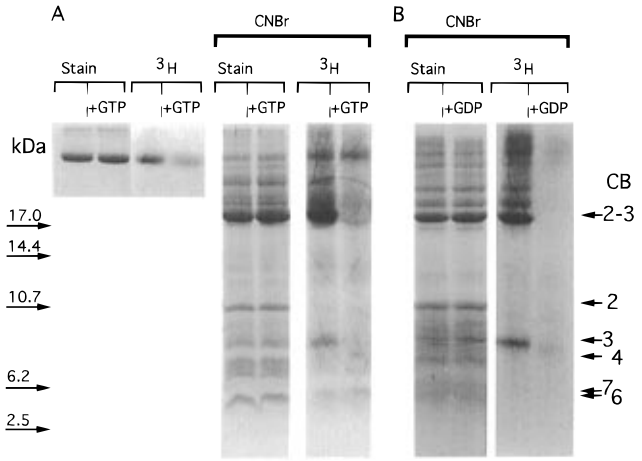

FIGURE 4: Reaction of WRK with isolated UCP monitored by labeling with [^{3}H]$NaBH_4$. SDS−PAGE of intact UCP and of CNBr cleavage products, Coomassie blue and autoradiography, with and without protection against incorporation of WRK (A) by GTP and (B) by GDP. Isolated UCP was labeled and tritiated with [^{3}H]$NaBH_4$ as described under Materials and Methods. For cleavage, UCP was precipitated with acetone/trichloroacetic acid and dissolved in 80% formic acid. The cleavage was carried out as described under Materials and Methods. 6 μg of uncleaved UCP and 50 μg of CNBr-cleaved UCP were applied to the lanes of a 16% double cross-linked polyacrylamide gel (described under Materials and Methods). For the detection of the [^{3}H]-labeled peptides, the stained and photographed gel was treated with Amplify, a fluorographic reagent, and dried under vacuum. The dried gel was autoradiographed with Hyperfilm-MP for 2 days at −70 °C. Small cracks in the Amplify-treated gels are nearly unavoidable.

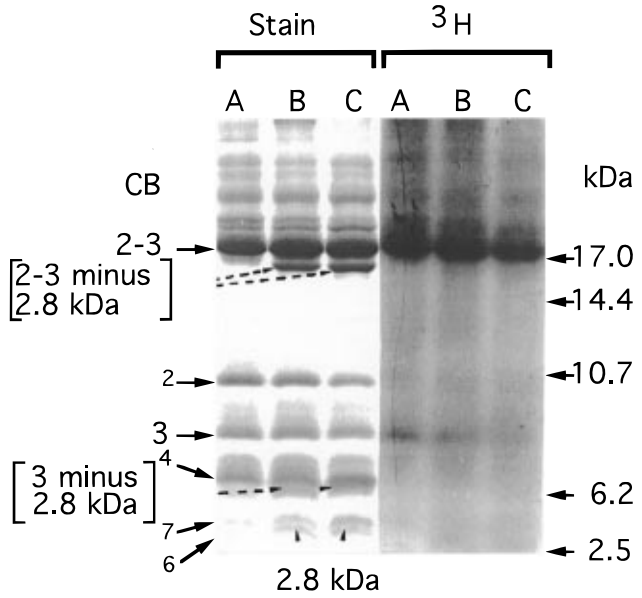

FIGURE 5: Combined CNBr and BNPS cleavage identifies a 2.8 kDaa fragment as the major WRK target region as measured by [^{3}H] incorporation. SDS−PAGE, Coomassie blue and autoradiography. Isolated UCP was reacted with WRK and labeled with [^{3}H]-$NaBH_4$ as described under Materials and Methods. For cleavage, UCP was precipitated with acetone/trichloroacetic acid and dissolved in 80% formic acid. The cleavage was carried out: (lane A) by incubation with CNBr, (lane B) by incubation first with CNBr for 4 h and afterward with BNPS overnight, (lane C) by simultaneous incubation with CNBr and BNPS. 50 μg of the cleaved peptides was separated on a 16% double cross-linked polyacrylamide gel.

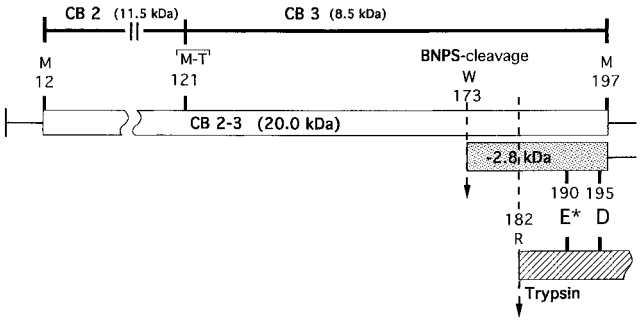

FIGURE 6: Scheme illustrating the various cleavage sites of UCP by CNBr and by the tryptophan reagent BNPS. A 2.8 kDa fragment is identified as the major [^{3}H]-labeled region. Furthermore, a cleavage site by trypsin located in this fragment is shown.

of [^{3}H] incorporation in the presence of GTP in most peptides. In CB3 the inhibition by GTP is nearly complete.

According to our model of the pH dependence of nucleotide binding, both the nucleoside di- and triphosphates require one common carboxyl group (Klingenberg, 1988; Huang & Klingenberg, 1995), whereas in the binding of the triphosphates an additional H^+-dissociating group is involved. Therefore, we examined also the protection by GDP in order to assign the WRK-sensitive group to one or both of the proposed H^+-dissociating residues. As shown in Figure 4B, GDP protects as well or in this particular experiment even better than GTP. Obviously, the WRK targeted group is common to both the GTP and GDP binding sites.

For further delimiting the WRK target, it was necessary to cleave the CB3 peptide (see scheme in Figure 6). We chose W173 which can be oxidatively cleaved with the tryptophan reagent BNPS (Fontana, 1972). The cleavage by CNBr and BNPS was combined either by first cleaving with CNBr and then with BNPS or in the reverse sequence. The cleavage by BNPS is only partial even under optimized conditions but well-defined on the gels. As clearly shown in Figure 5, on addition of BNPS to the CNBr cleavage products, three new bands appear (lanes B and C), satellites below CB2−3 and below CB 4 and a band at 2.8 kDa. The small 2.8 kDa peptide is to be expected as the BNPS cleavage product W173 to M197 (Figure 6). The band below CB2−3 corresponds to the product obtained after separation of the 2.8 kDa and below CB4 to the cleavage product CB3 minus 2.8 kDa. The stain of the 2.8 kDa peptide is weak due to the poor retention of small peptides on fixing and staining the gel and further due to the low content of amino acids.

In the radioautogram after BNPS treatment, the absence of [^{3}H] label in the major cleavage product CB2−3 minus 2.8 kDa band is striking (lanes B and C). [^{3}H] label is also missing in the CB3 minus 2.8 kDa product; correspondingly the [^{3}H] label in the CB3 is decreased after BNP treatment. But a new [^{3}H] label appears at the 2.8 kDa band which is only weak for the above-mentioned reasons. Most of the further [^{3}H] seen in the radioautograms can be rationalized to be present in peptides which contain the 2.8 kDa peptide. These results clearly point to the 2.8 kDa peptide region as the major carrier of the [^{3}H] label originating from the WRK incorporation.

Since the 2.8 kDa can be identified with the double cleavage peptide between W173 and the CB3 terminus at M197, we are left with two candidates as WRK targets, E190 and D195 (Figure 6). For a final decision on which of the two carboxyl carrying residues is targeted by WRK, the sequencing of the incorporated radioactivity in suitable peptides was performed. For this purpose, the 2.8 kDa peptide resulting from double cleavage of CNBr and BNPS

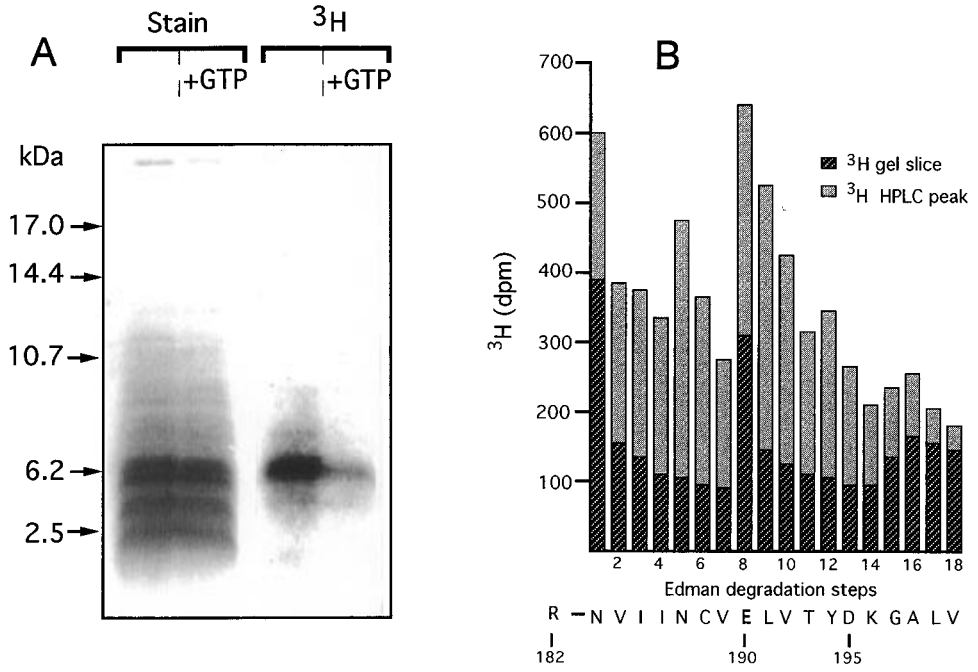

FIGURE 7: Preparation of [^{3}H]-labeled tryptic fragments of UCP reacted with WRK and sequencing by Edman degradation. (A) SDS−PAGE, Coomassie blue staining, and autoradiography. Isolated UCP, and as a control UCP preincubated with GTP, were reacted with WRK, then [^{3}H] labeled with [^{3}H]$NaBH_4$, and digested with trypsin as described under Materials and Methods. 42 μg of the peptides was applied to a 16% double cross-linked polyacrylamide gel (described under Materials and Methods). (B) Edman degradation of peptides from [^{3}H]-containing gel slice and from [^{3}H]-containing HPLC peak fraction: The [^{3}H]-labeled peptide was eluted from SDS−PAGE and washed as described under Materials and Methods. The tryptic fragments were isolated by reversed-phase HPLC using an RP-8 column as described under Materials and Methods. The [^{3}H]-containing peak fractions were dissolved in 100% trifluoroacetic acid and applied to a DITC-glass membrane as described in Wachter et al. (1973). For radiosequencing, in the degradation cycle the conversion of anilinothiazolinone was omitted. The [^{3}H] content of each degradation step was determined by scintillation counting. Sequencing was done on a sequencer Model Procise 492 from Applied Bioindustries.

was unsuited because of the low yield and the difficulty in handling small peptides. Further, the distance from the *N*-terminus of 17 residues until the first candidate incorporation site is also too large because of the usual radioactivity bleeding during sequencing. On the other hand, only the narrowing down by the combined CNBr and BNBS cleavage of the WRK targeted residues to E190 or D195 permitted now to further use tryptic fragmentation and to isolate a fragment suitable for sequencing. The tryptic cleavage site closest to the candidate residue E190 is R182. The resulting peptide has only 8 residues to be sequenced before it reaches E190 and 13 steps for reaching D195. Under the provision that WRK incorporates mainly at E190 or D195, we could expect that among the many tryptic fragments obtained from UCP only those containing these residues also carry [^{3}H] radioactivity. In order to avoid the fragmentation by trypsin of UCP into a maze of small, not separable fragments (Mayinger & Klingenberg, 1992), a novel method to only partially unfold UCP was introduced by the use of the mild anionic detergent laurylsarcosine. In this detergent trypsin retains most of its activity and UCP is unfolded to an extent to allow cleavage at many of the candidates' sites. In fact, as shown in Figure 7, the main trypsin fragments have a molecular weight of 6.2 kDa and lower. The major amount of radioactivity is found at M_r = 6.2 kDa, and most importantly, this radioactivity is largely suppressed by preincubation of UCP with GTP prior to the exposure to WRK and [^{3}H]$NaBH_4$. Thus, the tryptic peptide at the 6.2 kDa would be a major candidate for an Edman degradation to identify the position of the [^{3}H] incorporation. The peptide was isolated either directly from the [^{3}H]-containing gel slice by excision and elution or by HPLC chromatography of the tryptic peptide mixture.

Sequencing of amino acids of the HPLC fraction and of the radioactivity, both of the gel extract and HPLC fraction, was performed separately. The amino acid sequencing was used to assure that extracted or purified fractions mainly contained the peptide starting with R183. The sequencing of [^{3}H] radioactivity produced the usual unspecific peak in the first step with the release of unbound or degraded and the following [^{3}H] radioactivity bleeding in all the probes. A clear maximum of [^{3}H] appeared in step 8, in peptides which were both extracted from the gels or purified by HPLC. Step 8 in the Edman degradation process corresponds to E190 and thus exactly to one of the two carboxyl groups already identified previously by the combined CNBr and BNPS cleavage as targets of the specific GTP-sensitive WRK incorporation, whereas no [^{3}H] peak appears at step 13 where D195 is degraded. These results clearly identify the intrahelical glutamate E190 as the target of the GTP-sensitive WRK incorporation.

DISCUSSION

The strong and unique pH dependence of nucleotide binding to UCP had been analyzed in great detail using radioactively labeled nucleotides and fluorescent DANSYL derivatives (Klingenberg, 1988; Huang & Klingenberg, 1995). From these studies, a model was proposed where the nucleotide binding site is under control of one or two H^+-dissociating sites in the UCP, depending on whether the diphosphates or the triphosphates are binding. The extrapolated p*K* of about 4.5 for the H^+-dissociating group control-

ling both the binding of di- and triphosphate suggested that it is provided by a carboxyl group. The higher p*K* of an additional H^+-dissociating group involved in controlling only the binding of triphosphate suggests involvement of either histidine or a carboxyl group.

WRK Interactions in UCP. The nucleotide binding has been previously shown to be inhibited by several reagents, by the cysteine reagents tetranitromethane and NEM in mitochondria (Rial & Nicholls, 1986), by the lysine reagent DABS (Kopecky et al., 1986), and by the arginine reagent butanedione (Katiyar & Shrago, 1989). In all these cases, relatively high reagent concentrations were used, and with DABS and tetranitromethane the affinity rather than the binding were concluded to be affected. The carboxyl reagent WRK is yet by far the most specific and effective inhibitor of the nucleotide binding. It vindicated our postulate of the involvement of a carboxyl group in the phosphate binding domain of UCP. The fact that WRK does not inhibit H^+ or Cl^- transport (Figure 3) again emphasizes previous conclusions (Kopecky at al., 1986; Rial & Nicholls, 1986; Katiyar & Shrago, 1989) that the nucleotide binding site is not in the H^+ translocation pathway and that nucleotides inhibit through a conformation change (Klingenberg, 1984; Huang & Klingenberg, 1995, 1996).

The high "affinity" of WRK as based on the inhibition of nucleotide binding to isolated UCP is very intriguing. Despite a total of 20 carboxyl groups present in UCP, WRK at a molar ratio to UCP of only 2:1 seems to preferentially incorporate into the GTP-sensitive site. The additional $\approx$1 WRK/UCP consumed can be expected to be distributed among the other 19 carboxyl groups in UCP. A reasonable explanation for the strong preference of E 190 is that WRK has a relatively high binding affinity, near or at the nucleotide binding site. It is known that anions, in particular sulfate, are effective competitors for the nucleotide binding to UCP so that the anionic binding to the nucleotide sites appears to be comparatively unspecific (Lin & Klingenberg, 1982; Huang & Klingenberg, 1995). It is therefore suggestive that the sulfonate group is instrumental for binding of WRK to the nucleotide binding site and thus to ensure its high specificity and local reactivity. This is also in line with our model that the controlling carboxyl group is directly located at the binding site for the phosphate domain of the nucleotide (Klingenberg, 1988). With this reasoning, it seems improbable that the protection against WRK is mediated primarily via the conformational change from a remote nucleotide binding site.

Among the mitochondrial carriers, WRK was found to inhibit the phosphate-H^+ cotransport in mitochondria and in reconstituted liposomes by increasing the K_M (Wolf et al., 1989). It was concluded that WRK did not block the phosphate-H^+ binding site, but acted indirectly through a conformational change. The best documented case of the WRK influence on a carboxyl group in a carrier is the anion transporter of human erythrocytes (Jennings & Anderson, 1987; Jenning & Al-Rhaiyel, 1988). Interestingly, here again sulfate is interacting with the targeted carboxylic groups, which requires protonation in order to compensate one of the two negative charges of sulfate in the sulfate/chloride exchange. WRK was used here at millimolar concentrations on the erythrocyte membrane. Also in mitochondria a large molar excess of WRK over UCP is required for complete inhibition of GTP binding (Figure 1A), because of numerous additional reactive carboxyl groups. There are only a few examples reported in the literature where WRK can be incorporated at concentrations nearly equimolar to the specific target site. Examples of high sensitivity toward WRK and the use of only slightly hyperstoichiometric amounts are the modifications and identification of essential carboxyl groups by WRK in acid phosphatase (Saini & Van Etten, 1979) and of uridine phosphorylase (Komissarov et al., 1995). Other cases of enzymes where WRK has been useful to identify essential carboxyl groups are endonuclease D (Tomme et al., 1992).

Our procedure of applying WRK and labeling the incorporation site with [^{3}H] follows the pioneering work of Jennings on the anion transporter of erythrocytes (Jennings & Anderson, 1987). He introduced the reduction by borohydride of the WRK adduct soon after the addition of WRK. As a result, the WRK is cleaved and a primary alcohol formed from the carboxyl group under incorporation of two stable [^{3}H] atoms. The great advantage of this procedure is the retention of the molecular weight of the peptides and their only marginal chemical modification so that they will appear in the gels and also in the chromatography near the same position as the unmodified peptides. Indeed in our experiments the autoradiography of the gels of the CNBr and BNPS fragments shows (Figure 4) that the [^{3}H]-labeled bands nearly coincide with the underlying peptide band, indicating that the carboxyl group has in fact been reduced to the alcohol.

The Functional Position of E190. It is interesting to note that E190 is an intrahelical carboxyl group and that it occurs only in UCP at this position among the mitochondrial carrier family structures known so far (Klingenberg & Nelson, 1995). It is not surprising that within the 2.8 kDa peptide obtained by combined CNBr and BNPS cleavage and identified to carry the major portion of [^{3}H] incorporation, only E190 and not D195 is [^{3}H] labeled. D195 is assumed to be a helix terminating negative group which occurs in striking regularity at the downstream end of the six transmembrane helices in UCP. Thus, it seems to have a more structural than functional role. We find the same pattern of helix terminal acidic groups also in other members of the mitochondrial carrier family, however often with less regularity.

Since the carboxyl group E190 is protected against the incorporation of WRK by both GTP and GDP, we can identify E190 with the postulated low p*K* carboxyl group which is the pH control factor, common to both the di- and triphosphates. With E190 we have identified the fourth residue in UCP, near or at the nucleotide binding site. All 4 residues connect to different atoms or domains of the nucleotide, and they delineate an extensive interaction area with UCP (Figure 8). Photoaffinity labeling with 2-azido ATP has targeted T264 (Mayinger & Klingenberg, 1992) and with 8-azido ATP T259 (Winkler & Klingenberg, 1992). Both are located in the hydrophilic matrix loop of the third domain. Fluorodinitrophenyl-ATP with the covalent group substitution at the 3′ position of the ribose incorporates into C253, also within the matrix loop of the repeat domain (Mayinger & Klingenberg, 1992). The phosphate moiety of the nucleotides extends into the second repeat domain of UCP, where according to our model of the pH regulation E190 controls the access to the phosphate moiety binding cleft (Lin & Klingenberg, 1982; Huang & Klingenberg,

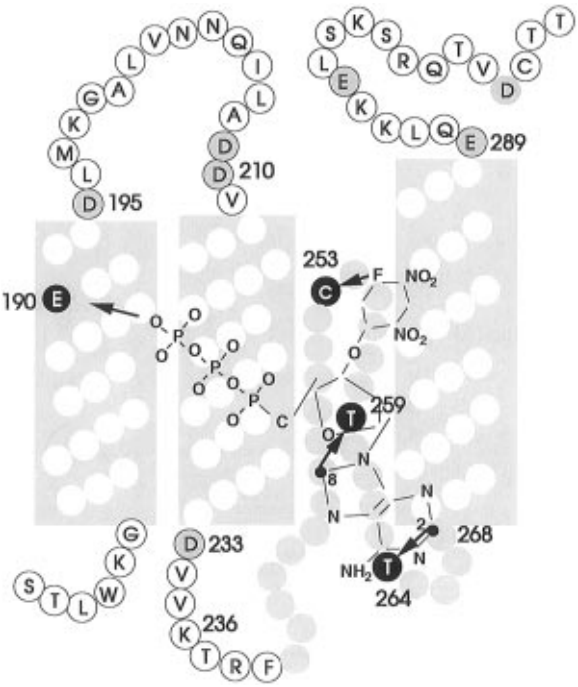

FIGURE 8: Scheme illustrating the four residues identified to be involved in nucleotide binding. E190 is the transmembrane folding of the second half of UCP.

1995). Although E190 is located within a transmembrane helix, it does not participate in the H^+ transfer through UCP as shown by the lack of H^+ transport inhibition on WRK treatment. It must however sense the external pH, either by free access to the cytosol, or indirectly, with the bulk pH relayed to E190 from another more at the surface located group, in a network of electrostatic interactions in UCP.

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