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Characteristics of the Isolated Purine Nucleotide Binding Protein from Brown Fat Mitochondria†

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ABSTRACT: The isolation of a purine nucleotide binding protein (NbP), the putative uncoupling protein, from hamster brown adipose tissue mitochondria and some of its functional characteristics are described. (1) Among various detergents tested, Triton is the most suitable; the total GDP binding capacity can be recovered after solubilization by Triton and is rather stable in this extract. (2) For separation of NbP from the ADP/ATP carrier, differences in the solubilizing conditions and the stability at room temperature between both proteins are exploited. The preparation is substantially free of ADP/ATP carrier. (3) The purified NbP has a binding capacity for 16 μmol of GDP/g of protein, corresponding to a 16-fold purification from mitochondria. (4) In sodium dodecyl sulfate–polyacrylamide gel electrophoresis a single band of M_r 32000 is found. A dimer structure is suggested from chemical cross-linking, from the binding capacity for GDP, and from the previously reported centrifugation equilibrium. (5) The isolated NbP preparation consists of Triton–protein–phospholipid mixed micelles with a Stokes radius of 60.5 Å as determined by gel filtration. The Triton binding is 1.9 g/g of protein, and the phospholipid binding is 0.2 g/g of protein. (6) The amino acid composition has a polarity index of 43.5%. The N-terminal peptide has the sequence Val-Asp-Pro-Thr-Thr-Ser-Glu-Val. (7) The affinity of NbP for different purine nucleotides decreases in the order GTP > GDP > ATP > ITP > ADP > IDP. The affinity for the monophosphates is 100 times lower. (8) Photooxidation and the lysine reagent 2,4,6-trinitrobenzenesulfonic acid decrease the binding capacity without influencing the affinity of the unaffected sites. GDP protects against photooxidation.

Mitochondria isolated from brown adipose tissue are largely uncoupled (Nicholls & Lindberg, 1973). Coupling can be restored by adding purine nucleotides (Hohorst & Rafael, 1968; Pedersen, 1970). These mitochondria possess an abnormally high permeability to protons as well as to chloride, bromide, and nitrate, which is decreased by purine nucleotides (Nicholls et al., 1974). A specific binding site for purine nucleotide was determined on the outer surface of the inner membrane of brown adipose tissue mitochondria (Rafael & Heldt, 1976; Nicholls, 1976). The number of these binding sites was found to increase during cold adaptation, corresponding to the thermogenic activity of brown adipose tissue (Rafael & Heldt, 1976; Sudin & Cannon, 1980).

In a study of polypeptide composition of rat brown adipose tissue mitochondria, Ricquier & Kader (1976) reported a prominent M_r 32000 component. Desautels et al. (1978) correlated the increase in binding of purine nucleotides during cold acclimation with the M_r 32000 component. By using 8-azido-[^{32}P]ATP,[1] Heaton et al. (1978) showed that the M_r 32000 component becomes the major labeled protein and that the labeling was prevented by a 10-fold excess of GDP (Heaton et al., 1978) but not by CAT. They claimed that the M_r 32000 component is the regulatory site of the energy-dissipating ion channel. Isolation of this protein was first reported by Ricquier et al. (1979) with GDP–agarose affinity chromatography. However, the purification was partial and with low yield.

The M_r 32000 protein has been tentatively referred to by us (Lin & Klingenberg, 1980a) as the "uncoupling protein" to emphasize its putative functional role in brown adipose tissue mitochondria. Recently the name "thermogenin" has been suggested (Lindberg et al., 1981). On the basis of the concept that NbP has certain similarities to the ADP/ATP carrier, the isolation method developed for the ADP/ATP carrier has been applied to NbP with some modifications, which are necessary to ensure the separation between the two proteins. This method was first briefly outlined for the isolation from cold-adapted hamster (Lin & Klingenberg, 1980a,b) and was then also applied for that purpose to cold-adapted rat (D. Ricquier, C. S. Lin, and M. Klingenberg, unpublished results). In view of the importance and growing interest in this protein a full account of the isolation procedures is given here. Furthermore, some characteristics of the isolated uncoupling protein are reported, in order to provide a basis for understanding its function.

Materials and Methods

Emulphogen BC 720 was obtained from GAF Co., New York, NY, [^{3}H]GDP, [^{14}C]ADP, and [^{14}C]ATP were from New England Nuclear, dimethylsuberimidate was from Pierce

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[1] Abbreviations: NbP, nucleotide binding protein; CAT, carboxyatractylate; DMS, dimethylsuberimidate; FCCP, carbonyl cyanide *p*-(trifluoromethoxy)phenylhydrazone; Mops, 3-(*N*-morpholino)propanesulfonic acid; NEM, *N*-ethylmaleimide; ATP, adenosine 5′-triphosphate; ADP, adenosine 5′-diphosphate; AMP, adenosine 5′-phosphate; GTP, guanosine 5′-triphosphate; GDP, guanosine 5′-diphosphate; GMP, guanosine 5′-phosphate; ITP, inosine 5′-triphosphate; IDP, inosine 5′-diphosphate; IMP, inosine 5′-phosphate; Tris, tris(hydroxymethyl)aminomethane; EDTA, ethylenediaminetetraacetic acid; $NaDodSO_4$, sodium dodecyl sulfate.

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Co., lactoperoxidase was from Boehringer, and Rose Bengal and 2,3,6-trinitrobenzenesulfonic acid were from Sigma.

Preparation of Mitochondria. Three-week-old gold hamsters were exposed to 4 °C for 3–4 weeks with food and water ad libitum. Interscapular, subscapular, axillary, and dorsal cervical brown adipose tissues were excised at 4 °C and put immediately into ice-cold 0.3 M sucrose containing 10 mM Tris-HCl, pH 7.2, and 2 mM EDTA. Muscle connective tissue and white fat were cut off.

For the preparation of mitochondria and for most incubations of the isolated protein the following "standard buffer" was used consisting of 20 mM Mops, 20 mM Na_2SO_4, and 1 mM EDTA, pH 6.7. For the isolation of mitochondria, tissue from four hamsters was homogenized in 75 mL of standard medium and then centrifuged at 700*g* for 10 min. The supernatant below the fat layer was sucked out carefully and filtered through gauze. The filtrate was spun at 6000*g* for 10 min. The supernatant was decanted, and fat adhering to the wall of the centrifugation tubes was swept with filter paper. The pellet was resuspended and again centrifuged at 6000*g*. This sediment was suspended at a concentration of 100 mg of protein/mL of standard buffer and stored in liquid nitrogen.

The protein content was determined by the method of Lowry in the presence of 1% $NaDodSO_4$ using bovine serum albumin as the standard. Phosphorus was estimated according to the method of Chen et al. (1956). Cytochrome oxidase content was estimated from the reduced (5 μM FCCP plus dithionite) minus oxidized (5 μM FCCP) difference spectra. The millimolar extinction coefficient used was $\epsilon_{605nm} = 24\ cm^{-1}$.

Determination of Binding. Binding of nucleotides was determined by equilibrium dialysis using a "Dianorm" apparatus (Dr. Weder, ETH, Zürich) with labeled nucleotides. The binding of unlabeled nucleotides was determined from competition with [^{3}H]GDP and evaluated according to

$$K_D^N = \frac{K_D^{GDP}[N]}{K_D'^{GDP} - K_D^{GDP}}$$

where K_D^N is the dissociation constant of the unlabeled nucleotide, [N] is the concentration of the unlabeled nucleotide, and K_D^{GDP} and $K_D'^{GDP}$ are the dissociation constants of GDP in the absence and presence of the unlabeled nucleotide N. The equation implies a simple competition between N and GDP.

Polyacrylamide slab *gel electrophoresis* was carried out as described by Douglas et al. (1979) with a gradient containing 10–15% acrylamide monomer. The gel was 1.5 mm thick and contained 0.1% $NaDodSO_4$. With this gradient a high resolution in the region M_r 25 000–40 000 was obtained. The gels were cast in special chambers constructed to permit filling through an opening from the bottom, which gave improved gradients.

Photooxidation was carried out at 0 °C. The mixture contained 10 μM freshly prepared Rose Bengal, 0.6–0.8 mg/mL NbP purified only up to the stage of hydroxylapatite column chromatography, 20 mM Mops, pH 6.7, 20 mM Na_2SO_4, and 0.16 mM EDTA. Samples were illuminated with a 150-W halogen tungsten lamp from the bottom in a modified Warburg apparatus under shaking. At the time indicated, 200 μL of the solution was withdrawn for equilibrium dialysis.

Results

Content of NbP in Cold-Adapted Brown Adipose Tissue Mitochondria. Cold-adapted brown adipose tissue mitochondria are rich in NbP. Data on the content of NbP as well as on the amount of cytochrome oxidase and ADP/ATP carrier protein are listed in Table I. The content of cytochrome aa_3 is at the same level as that in beef heart mitochondria (Klingenberg, 1978). The amount of ADP/ATP carrier protein as reflected in the binding capacity for [^{3}H]-CAT is considerably lower in the mitochondria from brown adipose tissue than that from beef heart [1.2–1.6 μmol/g of protein, cf. Klingenberg (1978)]. The molar ratio of the GDP binding capacity to the content of cytochrome aa_3 reaches 2.5. Thus in terms of molar content, NbP seems to be one of the most abundant proteins in brown adipose tissue mitochondria.

Table I: Purine Nucleotide Binding Protein (NbP) and ADP/ATP Carrier Protein Content in Brown Adipose Tissue Mitochondria of Cold-Adapted Hamsters[a]

	content (μmol/g of protein)
cytochrome aa_3	0.41 ± 0.04
NbP	1.02 ± 0.13
NbP-cytochrome aa_3	2.49
ADP/ATP carrier protein	0.35 ± 0.08
ADP/ATP carrier protein-cytochrome aa_3	0.85

[a] The content of NbP and ADP/ATP carrier was determined by measuring the binding capacity with the method of equilibrium dialysis. The incubation medium contained 12 mg of mitochondrial protein/mL and [^{3}H]GDP or [^{3}H]CAT. Equilibrium dialysis was carried out at 4 °C for 5 h at pH 6.7.

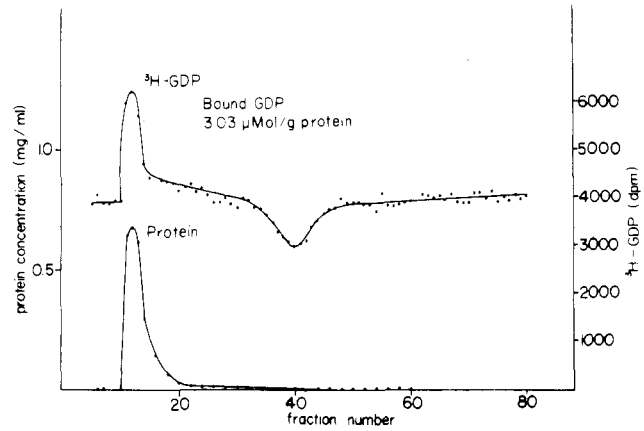

FIGURE 1: Binding of [^{3}H]GDP to solubilized NbP, as measured by gel filtration. Exclusion chromatography on [^{3}H]GDP equilibrated column: 300 μL of Triton extract of mitochondria (2 mg of protein) was mixed with 15 μL of 0.2 mM [^{3}H]GDP and then applied to a 1 × 30 cm Sephadex G-75 column that was preequilibrated with 5 μM [^{3}H]GDP in standard buffer at 4 °C. Elution was made with the same buffer in 0.5-mL fractions, at a flow rate of 8 mL/min.

Solubilization by Detergents. The specific binding capacity of the NbP for purine di- and triphosphate nucleotides can serve as a suitable assay for this protein after solubilization and purification. It has previously been shown that the specific binding, as originally observed on mitochondrial membranes, is retained after solubilization in certain detergents (Ricquier & Kader, 1976). Two chromatographic methods were used to assay the binding of GDP to the solubilized protein in the 100000*g* supernatant of the crude Triton X-100 extract. In the most simple procedure the supernatant was preincubated with [^{3}H]GDP and then filtered through Sephadex G-75 (not shown). However, in this case, because of the relatively high dissociation constant, part of GDP has been lost. Binding was more accurately determined by elution chromatography (Figure 1). In both methods, GDP binding was observed, giving proof that the solubilized NbP retains its GDP binding capacity. The concentration of GDP (5 μM) is not saturating,

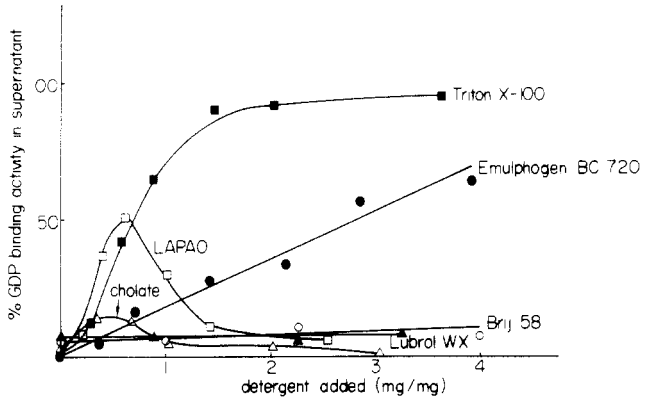

FIGURE 2: Effect of different detergents on the solubilization of NbP. Mitochondria were incubated in a medium containing different concentrations of detergents in standard buffer for 30 min at 0 °C. The 100000*g* supernatant was taken for measuring GDP binding. The binding capacity of intact mitochondria in the absence of detergent was referred to as 100%. The binding was determined by equilibrium dialysis (see Materials and Methods). LAPAO, 3-lauramido-*N*,*N*-dimethylpropylamine oxide.

and only limited binding is achieved. For quantitative analysis the equilibrium dialysis method is superior by which the binding of GDP has been demonstrated previously (Lin & Klingenberg, 1980a).

The isolation of NbP affords another interesting excercise for the use and classification of various detergents in the isolation of intact membrane protein. By use of the binding for GDP as an assay for solubilization and preservation of intactness, various nonionic detergents and cholate were tested (Figure 2). Only two poly(oxyethylene)-type detergents, Triton X-100 and Emulphogen BC 720, produced satisfactory results. Triton X-100 is the most suitable detergent, as it solubilizes NbP and retains the binding capacity. Emulphogen also preserves the binding capacity but is a less efficient solubilizer. It has the advantage over Triton in that it does not absorb light in the aromatic spectral region and is therefore useful for certain spectroscopic studies of the protein. The linear alkyl poly(oxyethylene) detergents Brij 58 or Lubrol WX are unable to solubilize the inner mitochondrial membrane. On the other hand, amine oxide type detergents such as 3-lauramido-*N*,*N*-dimethylpropylamine oxide are more powerful solubilizers than Triton but have a harsher effect, as evidenced by the decrease of GDP binding capacity. For example, at 1.8% 3-lauramido-*N*,*N*-dimethylpropylamine oxide or at 0.4% dodecyldimethylamine oxide, the binding activity is half suppressed. A similar graduation of the inoffensiveness of detergents had been obtained with the ADP/ATP carrier. However, with NbP no effective protection by the inhibitor ligand from denaturation by the more offensive detergents is possible. This is reasonably explained by the less tight binding of the nucleotide to NbP as compared to that of CAT to the ADP/ATP carrier.

NbP was previously reported (Lin & Klingenberg, 1980a) to be more easily detached from mitochondria than the ADP/ATP carrier. A lower amount of Triton is required and—most strikingly—nearly no salt is necessary in addition to the detergent, whereas solubilization of the ADP/ATP carrier is strongly stimulated by increasing ionic strength (Klingenberg et al., 1978). Therefore, if appropriate amounts of Triton are choosen and low ionic strength is maintained, the soluble extract can contain a higher ratio of NbP to ADP/ATP carrier than present in the original mitochondria.

As judged from the GDP binding capacity, the solubilized NbP is rather stable in the crude Triton extract. The loss of GDP binding activity at various temperatures was determined

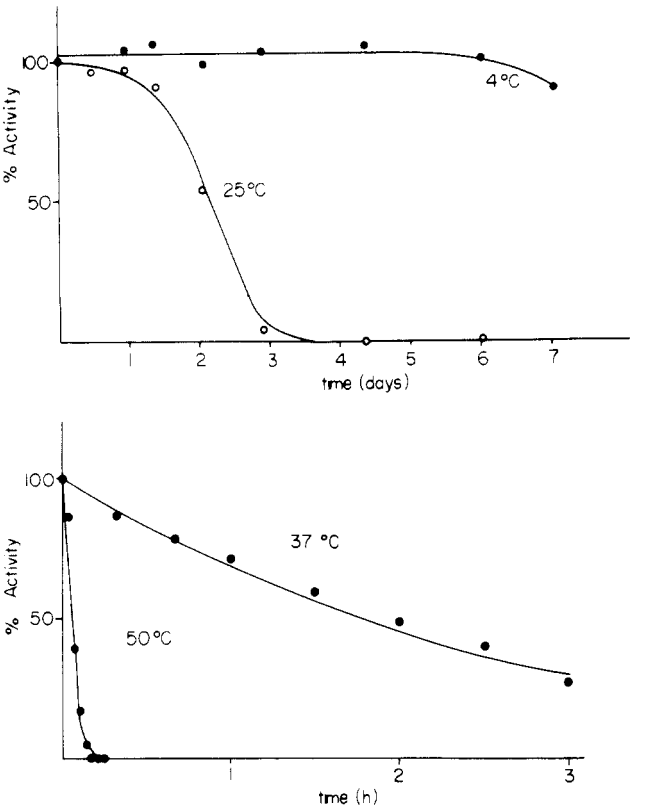

FIGURE 3: Stability of GDP binding capacity in the Triton extract from mitochondria at different temperatures. Mitochondria were incubated at 0 °C in a buffer containing 4% Triton, 15 mM Mops, 15 mM Na_2SO_4, 0.11 M sucrose, and 0.4 mM EDTA for 30 min. After centrifugation at 100000*g* for 30 min, the supernatant was kept at different temperatures. At the time indicated, a portion was withdrawn and the GDP binding capacity was measured by equilibrium dialysis. The binding capacity right after centrifugation was referred to as 100%.

as a function of time as shown in Figure 3. Binding capacity is retained at 0 °C for at least 7 days and at room temperature for only 1–2 days. In liquid nitrogen, NbP has been stored for more than 14 months without a significant change in binding capacity.

Purification. Before extraction of the NbP, soluble proteins and those peripheral to the membranes can be removed by treatment with Lubrol WX or Brij 58. The residual sedimented membranes should be washed free of Lubrol by resuspension in the sucrose medium in order to use "minimum" amounts of Triton for the subsequent solubilization. Otherwise, remaining Lubrol sequesters Triton by forming mixed micelles, and consequently more Triton would be needed. Chromatography on hydroxylapatite is a major purification step. The Triton extract of mitochondria is applied directly to the column, and a rapid and high degree of purification is obtained. NbP is confined to the breakthrough whereas most of the other solubilized proteins are adsorbed. For elimination of ADP/ATP carrier protein, the chromatography is run at room temperature. The unprotected ADP/ATP carrier protein is relatively easily denatured and decomposed and then seems to be absorbed on hydroxylapatite.

The enriched extract was further purified by sucrose gradient centrifugation. The concentrated extract was applied to a 8–20% linear sucrose gradient and centrifuged in a vertical tube rotor. This treatment also has the advantage that excess Triton and extracted phospholipid are separated from the protein. The final preparation after the sucrose gradient step binds about 16 μmol of GDP/g of protein. This corresponds to a total of 16-fold purification. A further attempt to purify

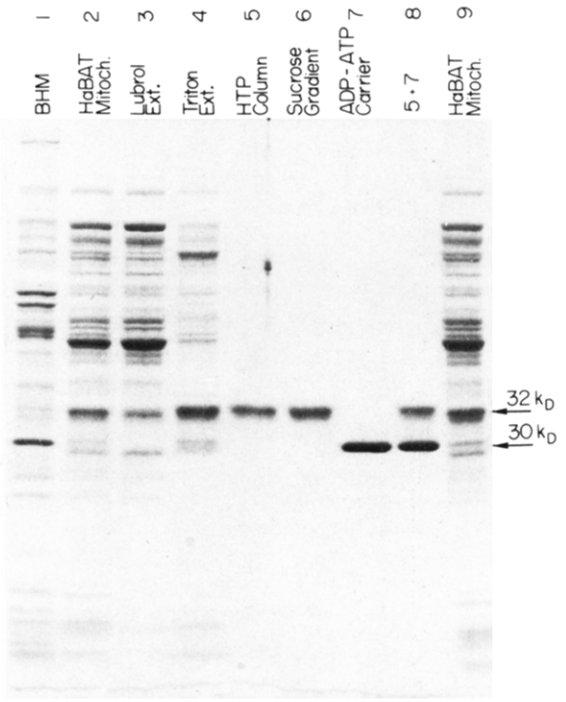

FIGURE 4: Gradient polyacrylamide slab gel electrophoresis in Na-DodSO$_4$. From left to right: (1) beef heart mitochondria (BHM), (2 and 9) hamster brown adipose tissue mitochondria (HaBAT), (3) Lubrol extract, (4) Triton extract, (5) hydroxylapatite column pass-through fraction (HTP column), (6) sucrose gradient fraction, (7) ADP/ATP carrier from beef heart mitochondria, and (8) hydroxylapatite column pass-through fraction plus ADP/ATP carrier.

NbP by chromatography on agarose (AcA 34 Ultrogel) did not increase the specific binding.

The qualitative assay for the purification by gradient Na-DodSO$_4$ gel electrophoresis is shown in Figure 4. The M_r 32000 band, corresponding to NbP, is the main band already in the crude extract. After hydroxylapatite chromatography, only one band is observed, and also the peak fractions after sucrose gradient centrifugation give a single protein band with only a faint shoulder of slightly lower molecular weight. The M_r 32000 band is conspicuously diffuse. Probably, this does not reflect impurities but heterogeneous unfolding and binding to the protein by NaDodSO$_4$. Possibly also the shoulder is part of this heterogeneity and not due to an impurity. The same shoulder is found in the original mitochondria and in all purification stages. Furthermore, N-terminus determination (see below) indicates that we are dealing with a single protein.

The coelectrophoresis of the purified NbP and purified ADP/ATP carrier from beef heart mitochondria gives a clear-cut separation and also shows that there is no substantial amount of ADP/ATP carrier in the NbP preparation unless the impurity has a blocked N terminal. Also the binding capacity indicated that the final preparation is more than 90% pure, when a half-site reactivity is assumed (see Lin & Klingenberg, 1980a).

The *standard purification procedure* derived from these experiments is the following: Brown adipose tissue mitochondria from hamster (0.5 g of protein) are first treated with 18 mL of the standard medium containing 3.2% Lubrol WX for 30 min at 0 °C. The standard medium consists of 20 mM morpholinopropane sulfonate, 20 mM Na_2SO_4, and 0.16 mM EDTA, pH 6.7. The suspension is centrifuged at 100000*g* for 30 min. The sediment is resuspended with 0.3 M sucrose, 10 mM Tris, and 2 mM EDTA, pH 7.2, and again centrifuged at 100000*g* for 30 min. This sediment is then resuspended in 16 mL of standard medium, containing 5% Triton X-100, for 30 min at 0 °C. The suspension is again centrifuged at 100000*g* for 30 min.

The supernatant is applied to a hydroxylapatite column (3 × 10 cm) that was equilibrated with standard medium. The protein is eluted at room temperature with the same medium. The breakthrough fractions are pooled and concentrated by pressure dialysis to 5–8 mL. This solution is applied in two to three portions of 2 mL on 35 mL of a 8–20% linear sucrose gradient in standard medium, with the addition of 0.1% Triton X-100. After centrifugation in the "50-VTi" vertical rotor for 14 h at about 160000*g*, 2.5-mL fractions are collected. The fractions with high binding capacity for GDP are pooled, dialyzed, and concentrated. The final preparation of about 3 mL containing 8–12 mg of protein is stored at −20 °C.

Binding Properties. The binding of purified NbP is highly specific for the di- and triphosphate derivatives of purine nucleotides as shown in Table II. The affinities do not differ much and are highest for the guanosine derivatives and lowest for the inosine derivatives. The affinity of the monophosphates is lower by 2 orders of magnitude. Obviously, the negative charges of the pyrophosphate moiety for the nucleotides play an important role in binding.

The binding of purine nucleotides for NbP is pH dependent (Figure 5). The plot of the pK_D against pH gives two important parameters. There is a break in the pH dependence at 6.4, and beyond that point the pK_D decreases with

Table II: Binding Specificity of NbP[a]

	K_D (μM)	binding capacity (μmol/g of protein)
GTP	1.4	15.1
GDP	2.5	16.2
ATP	3.1	14.7
ITP	4.7	16.9
ADP	4.9	16.5
IDP	8.0	14.5
GMP	110	17.4
AMP	424	14.2
IMP	730	15.1
		15.6 ± 1.14 [b]

[a] Binding was determined by equilibrium dialysis in standard medium, containing 0.5–0.7 mg of NbP/mL. In the case of GDP, ADP, and ATP, K_D and binding capacity were obtained directly from a mass action plot by using radioactive nucleotides, respectively. For the remaining nucleotides, binding was determined from competition with [^{3}H]GDP (for an evaluation, see Materials and Methods). [b] Average.

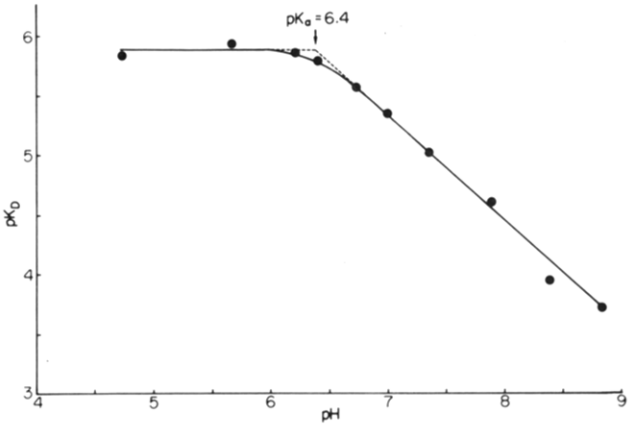

FIGURE 5: Influence of pH on GDP binding. NbP protein was purified to the step of hydroxylapatite column chromatography. The K_D at each pH was evaluated from a mass action plot. GDP binding was determined by equilibrium dialysis in a medium containing 25 mM succinate, 30 mM Mops, 25 mM Tris, 10 mM Na_2SO_4, and 0.16 mM EDTA.

Table III: Inhibition of GDP Binding to Brown Adipose Tissue Mitochondria by Salts[a]

	salt concn for 50% inhibn of GDP binding (mM)
NaCl	340
NaBr	320
$NaNO_3$	240
NaH_2PO_4	200
imidazole	180
Na_2SO_4	140
$Na_4P_2O_7$	25

[a] Frozen-thawed mitochondria with no osmotic barrier were used. Binding of GDP was determined in the presence of different concentrations of salts. The salt concentration for 50% inhibition of GDP binding was estimated from the curve of binding vs. salt concentration. The incubation contained 20 mM Mops, pH 6.7, 10–14 mg of mitochondrial protein/mL, 100 mM sucrose, and the different concentrations of salts.

Table IV: Amino Acid Composition of NbP from Brown Adipose Tissue Mitochondria of Cold-Adapted Hamsters in Mole Percent[a]

Asx	6.1	Cys	1.6	Pro	5.0	Tyr	3.3
Thr	9.9	Met	1.9	Gly	8.7	Phe	5.4
Ser	7.2	Ile	5.7	Ala	7.0	His	2.6
Glx	8.8	Leu	10.4	Val	7.4	Lys	5.0
						Arg	3.9

polarity: 43.5

N-terminal peptide:

Val-Asx-Pro-Thr-Thr-Ser-Glu-Val-?-Pro-Thr-Met-Gly-Val

1 2 3 4 5 6 7 8 9 10 11 12 13 14

[a] Purified NbP was precipitated by 5% trichloroacetic acid in 50% acetone. The precipitate was washed twice by aqueous acetone and was dissolved in 80% formic acid. Sequencing was done by an automatic liquid-phase apparatus (Beckman Sequencer 890C) with 50 nmol of protein, giving about 50% yield of the N-terminal values, corresponding to a usual yield obtained in this laboratory with pure protein. The PTH derivations were quantitatively determined by an HPLC system by using automatic integration (H. Aquila, unpublished results).

$\Delta pK_D/\Delta pH = -1$. This indicates the involvement of an ionizable group with $pK_D = 6.4$ in the binding. A more detailed analysis of the pH dependency is presently under way.

Salts inhibit the binding of purine nucleotides to NbP in the isolated as well as in the original membrane-bound state. The binding of GDP to NbP is diminished by increasing Na_2SO_4 concentration with 50% inhibition at about 80 mM Na_2SO_4. Binding of GDP to the mitochondria is somewhat less influenced by Na_2SO_4, as shown in Table III, where various salts are compared. Studies of the concentration dependence (not shown) both on the soluble NbP or on membrane-bound NbP demonstrate a competitive inhibition by salts and no change of the binding capacity. It appears that the inhibition is caused by competition with GDP of the anions for the cationic charges at the GDP binding site. Divalent anions are more effective than the monovalent halogenides. Pyrophosphate inhibits considerably stronger, probably by binding to the site for the pyrophosphate moiety in the nucleotides.

Molecular Properties. The amino acid composition of the purified protein is given in Table IV. The protein does not have a particular excess of hydrophobic amino acids, and the polarity index, calculated according to Capaldi & Vanderkooi (1972), is 43.5%. The amino acid composition is not similar to that of the ADP/ATP carrier. The high content of threonine is striking. The N-terminal residue is valine; the N terminus is blocked in the ADP/ATP carrier. The N-terminal sequence has been determined up to 14 residues.

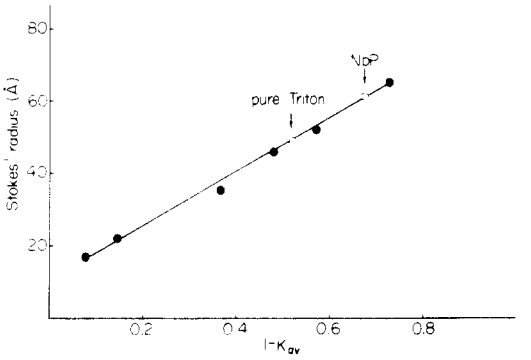

FIGURE 6: Determination of Stokes radius by gel chromatography. A 1 × 100 cm Ultrogel AcA-34 column was equilibrated with standard medium plus 0.1% Triton X-100 at 4 °C. The exclusion volume as determined with blue dextran is 24.05 mL. The total volume as measured with [^{3}H]sucrose is 81.41 mL. The Stokes radii of calibration protein were as follows: ferritin, 65 Å; catalase, 52 Å; aldolase, 46 Å; bovine serum albumin, 35 Å; chymotrypsinogen, 22 Å; cytochrome *c*, 17 Å.

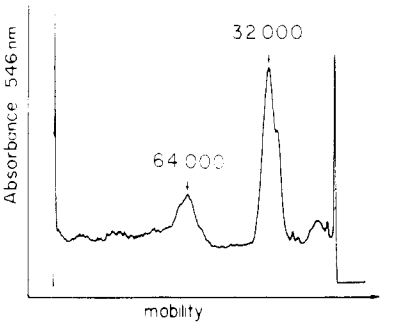

FIGURE 7: Cross-linking of purified NbP by dimethylsuberimidate. Protein (1.4 mg/mL) was reacted with 7.6 mM dimethylsuberimidate in 90 mM triethanolamine, pH 8.5, at room temperature. After 1 h the reaction was stopped by adding HCl to bring the pH to 6.5.

The purified NbP binds a large amount of Triton. The mixed protein micelle appears to exist in the form of monodisperse Triton–phospholipid–protein mixed micelles, as shown previously by hydrodynamic studies of the purified protein (1.9 Triton X-100/g of protein) (Lin et al., 1980). The residual binding of phospholipid was determined to be 0.2 g/g of protein. By adding an excess of Triton, amounting to 16 times the amount of Triton present in the purified preparation, and a subsequent second sucrose gradient centrifugation, the phospholipid content could be further reduced to 0.06 g/g of protein.

The Stokes radius of the isolated protein–Triton micelle was determined by gel filtration as shown in Figure 6. In a carefully calibrated curve $R_S = 60.5$ Å is evaluated.

On the basis of the GDP binding of the purified protein and in particular from ultracentrifugation studies, it was concluded that solubilized NbP is a dimer (Lin & Klingenberg, 1980a; Lin et al., 1980). Further evidence for the dimeric structure can be derived from cross-linking studies. As shown in Figure 7, after reaction with DMS about 25% of NbP exhibits an M_r of 64 000 in $NaDodSO_4$ gel electrophoresis. The yield of the dimer formation is unchanged if the concentration of NbP is reduced 10-fold. This indicates that cross-linking is not caused by bridging two colliding monomers but by internal cross-linking of a preexisting dimer. The fact that only $^1/_4$ of NbP can be cross-linked may be attributed to a high proportion of intramonomer cross-linking. The same has been observed for the isolated ADP/ATP carrier (Hackenberg & Klingenberg, 1980). This may also be the explanation for the peak broadening of the remaining monomers in the gel.

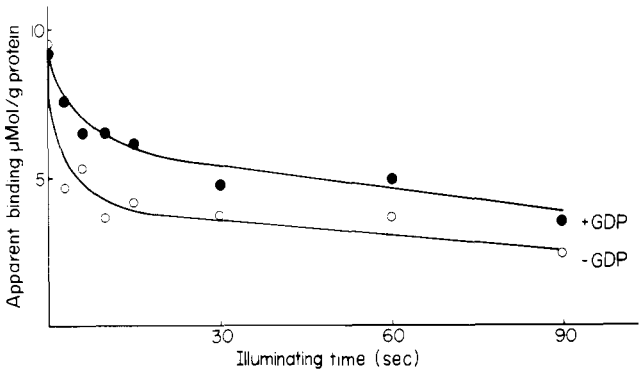

FIGURE 8: Loss of GDP binding capacity by photooxidation. Protection by GDP (27 μM). For experimental details see Materials and Methods.

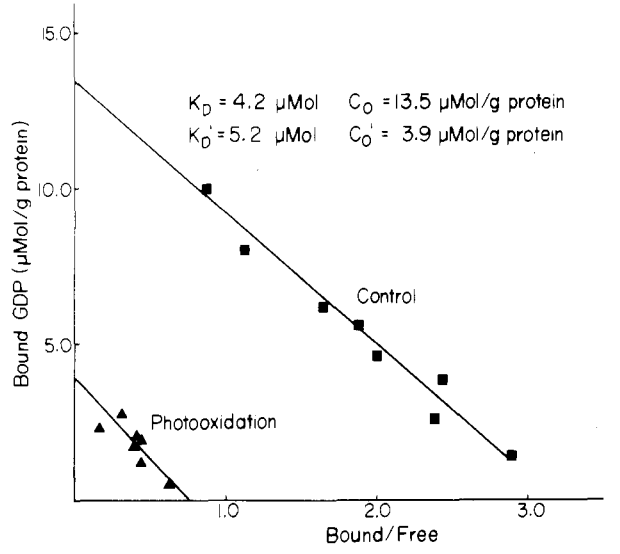

FIGURE 9: Mass action plot of GDP binding before and after photooxidation. NbP was purified to the step of hydroxylapatite column chromatography.

Chemical Modification of NbP. Binding of nucleotides to NbP is sensitive to dye-sensitized photooxidation. As shown in Figure 8, nearly 75% binding activity was lost during a 90-s illumination. GDP protects against binding inactivation by photooxidation, although not completely. The mass action plot shows clearly that photooxidation decreased the total binding sites but did not alter the dissociation constant (Figure 9). It is reasonable to assume that a photooxidizable amino acid residue in NbP is responsible for the binding of GDP.

The photooxidation of histidine is said to be sharply pH dependent (Westhead, 1972) as the pronated form of histidine is resistant to oxidation. However, careful analysis of the photoinactivation in the pH ranges from 5.4 to 8.8 gave no indication of pH influence. Therefore, it seems unlikely that histidine would participate in the binding of GDP. There is no influence of dithiothreitol on the rate of photooxidation. The participation of cysteine or methionine in the binding is therefore quite improbable. SH groups are photooxidizable, especially above pH 8 (Westhead, 1972). Moreover, cysteine as well as methionine can be protected with reducing agents (Westhead, 1972). Experiments using alkylating SH reagents such as NEM show that the binding of GDP is rather resistant to NEM. The possibility that photooxidizable tryptophan may be responsible for the photoinhibition of binding remains open.

That the binding center of NbP contains cationic amino acid residues is to be expected in view of the binding of highly negatively charged purine nucleotides. The predominantly lysine specific reagent 2,4,6-trinitrobenzenesulfonic acid inhibits the binding of GDP to NbP. The inhibitory effect on the binding of [^{3}H]GDP is observed, both on brown adipose tissue mitochondria (Figure 10) and on isolated NbP (not shown). The mass action plot reveals that 2,4,6-trinitrobenzenesulfonic acid reduces the binding capacity without changing the K_D of the surviving sites. It can be inferred that the participation of lysine in the binding of GDP is quite probable. The arginine reagent phenylglyoxal is considerably less effective. Only 30% inhibition can be reached at extensive exposure to this reagent (not shown).

Discussion

The purification procedure for NbP of brown adipose tissue in mitochondria resembles closely the procedure developed in this laboratory for purification of the ADP/ATP carrier from beef heart mitochondria (Riccio et al., 1975; Klingenberg et al., 1978). In this respect the presupposition of a similarity between both proteins has been very fruitful. It seems now paradoxical that this presumption was first a barrier for the isolation of NbP because a priori the separation of the two proteins seemed to be difficult unless a specific differentiation was possible. For this reason Ricquier et al. (1979) used GDP affinity chromatography, which was supposed to discard the ADP/ATP carrier. However, the inherent difficulties in that procedure such as low capacity and easy degradation of the affinity column could be avoided by using the unspecific, high-capacity procedures as described here. In fact, this simple, three-step procedure yields a preparation essentially free of ADP/ATP carrier, as judged from gel chromatography, amino acid composition, and CAT binding assay.

There are three reasons for the virtual absence of the ADP/ATP carrier from NbP preparation. NbP is in a 3-fold excess to ADP/ATP carrier in the brown adipose tissue of mitochondria. The extraction conditions favor NbP over ADP/ATP carrier. Without protection by CAT, the ADP/ATP carrier is decomposed comparatively rapidly at the room temperature used for solubilization and hydroxylapatite chromatography. Moreover, decomposed carrier protein is absorbed on hydroxylapatite (Klingenberg et al., 1978).

The nonadsorptivity to hydroxylapatite of NbP is a remarkable feature in common with the ADP/ATP carrier and apparently also with the P_i carrier (Wohlrab, 1980). Extensive shielding of the protein by a Triton envelope has been suggested to be responsible for the nonadsorptivity (Klingenberg et al., 1978) and, therefore, is typical also of other intrinsic membrane proteins, in particular, the metabolite exchange carriers in mitochondria. However, the protein unfolding on denaturation probably exposed polar surfaces, which originate from the interior and which may then adsorb to the hydroxylapatite.

Molecular Properties. It is remarkable that NbP exhibits structural features resembling closely those of the ADP/ATP carrier (Hackenberg & Klingenberg, 1980). First, the molecular weight is quite similar and marks a size for integral membrane proteins, which apparently is just sufficient to span the membrane. In addition to NbP, the ADP/ATP carrier and the P_i carrier of mitochondria (Wohlrab, 1980), and also bacteriorhodopsin (Ovchinnikov et al., 1979), have an M_r around 30 000.

Similar to the ADP/ATP carrier, the protein is a dimer and, most notably, exhibits half-site reactivity for its specific inhibitor ligand. The minimum functional M_r 60 000 derived from the binding capacity for nucleotides has been substantiated as the actual molecular weight of the protein by the centrifugal studies (Lin et al., 1980). Here, as in the ADP/ATP carrier, a high amount of Triton surrounds the

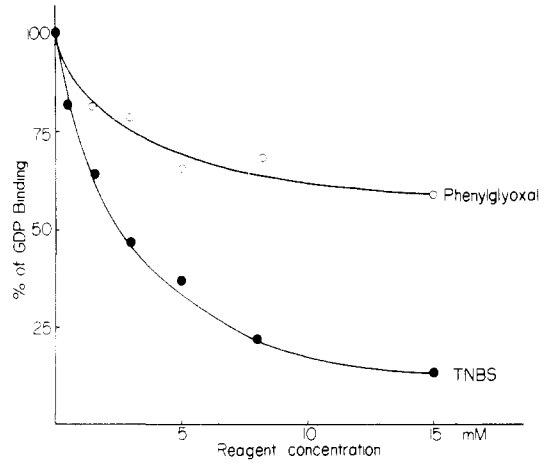

FIGURE 10: Inhibitory effect of 2,4,6-trinitrobenzenesulfonic acid (TNBS) and phenylglyoxal on the binding activity of NbP. 0.5 mg of mitochondria was incubated in 0.25 M sucrose and 20 mM Mops, pH 8.0, at 20 °C. Different concentrations of TNBS were added and reacted for 10 min. The reaction was stopped by adding HCl to bring the pH to 6.5. The GDP binding activity was then determined by equilibrium dialysis with 2.4 μM [^{3}H]GDP. The reaction with phenylglyoxal was conducted in a medium containing 40 mM borate, pH 7.6, at 20 °C.

protein, thus increasing the particle size to $M_r \simeq 170000$. A large surface of the protein seems to be covered by Triton molecules and, as judged from the frictional ratio, forms an oblate ellipsoid, which is spanned by the protein with its 2-fold axis along the short axis of the ellipsoid (Hackenberg & Klingenberg, 1980). According to the amino acid composition the protein has a moderate average polarity, slightly higher than that of the ADP/ATP carrier (Klingenberg et al., 1978). In fact, both proteins are relatively polar as compared to other intrinsic membrane proteins of the same size such as cytochrome *b*, the galactoside carrier, and bacteriorhodopsin. Obviously NbP must have an abundantly polar interior, encased by a hydrophobic jacket extending over the largest part of the surface facing the lipid bilayer.

The surprisingly high amount of NbP of mitochondria (6–8% of total mitochondrial protein and 14% of membrane protein) emphasizes its key role in the brown adipose tissue. There are about 2.6 molecules of NbP/molecule of cytochrome aa_3. Accordingly, each coupling site of the respiratory chain could be equipped with one molecule of NbP. NbP has been suggested to form channels of OH^- or other anions, thereby collapsing the membrane potential generated by electron transport (Nicholls et al., 1974). The nearly 1:1 molar ratio of NbP to coupling sites would agree with the concept of a "localized" H^+ generation at each site. However, this ratio is more accidental if one assumes that the NbP forms a channel for compensating "unlocalized" H^+. On the other hand, in this case the high content of NbP seems surprising, since the large amount of carriers for relatively small ions should represent a much higher capacity than required to compensate the production of about 10^3 H^+/min or OH^-/min per coupling site, at 37 °C, to be derived from respiratory activity.

The best analytical tools are the specific inhibitor ligands, purine di- and triphosphonucleosides, not only for identifying, isolating, and purifying NbP but also for analyzing its mechanism. It seems highly probable that, thus, nucleotides are regulators of the uncoupling function in the brown adipose cells. However, at the moment it is difficult to visualize, in view of the relatively high content of ADP and ATP, surpassing hundredfold the K_D of NbP, how the protein can be active at all. The uncoupling site should always be saturated from the outside, unless an additional mechanism diminishes the affinity.

Major questions as to the mechanistic function and to the regulation of NbP still remained unresolved. With isolated purified NbP it should be possible to approach these problems by incorporating the protein into phospholipid vesicles. There its functional properties can be studied, independently of the complications in the original mitochondria.

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