Beauchamp, P. M., Horn, E. W., & Gross, S. R. (1977) *Proc. Natl. Acad. Sci. U.S.A. 74*, 1172–1176.
Boguslawski, G., Vodkin, M. H., Finkelstein, O. B., & Fink, G. R. (1974) *Biochemistry 13*, 4659–4667.
Charezinski, M., & Borkowski, T. (1981) *Arch. Biochem. Biophys. 207*, 241–247.
Dang, C. V., & Yang, D. C. H. (1979) *J. Biol. Chem. 254*, 5350–5356.
Dang, C. V., & Yang, D. C. H. (1982) *Int. J. Biochem. 14*, 539–543.
Dang, C. V., Johnson, D. L., & Yang, D. C. H. (1982) *FEBS Lett. 142*, 1–6.
Deutscher, M. P. (1967) *J. Biol. Chem. 242*, 1123–1131.
Deutscher, M. P. (1974) *Methods Enzymol. 29*, 577–583.
Dickman, S. R., & Boll, D. J. (1977) *Biochem. Biophys. Res. Commun. 78*, 1191–1197.
Dietrich, A., De Marcillac, G. D., Pouyet, J., & Giegé, R. (1978) *Biochim. Biophys. Acta 521*, 597–605.
Holley, R. W., Apgar, J., Doctor, B. P., Farrow, J., Marini, M. A., & Merrill, S. H. (1961) *J. Biol. Chem. 236*, 200–202.
Irvin, J. D., & Hardesty, B. (1972) *Biochemistry 11*, 1915–1920.
Jeffrey, P. D., Shaw, D. C., & Treacy, G. B. (1976) *Biochemistry 15*, 5527–5533.
Joachimiak, A., & Barciszewski, J. (1980) *FEBS Lett. 119*, 201–211.
Kane, S. M., Vugrincic, C., Finbloom, D. S., & Smith, D. W. E. (1978) *Biochemistry 17*, 1509–1514.
Kellerman, O., Brevet, E., Tonetti, H., & Waller, J.-P. (1979) *Eur. J. Biochem. 99*, 541–550.
Kisselev, L. L., & Favorova, O. O. (1974) *Adv. Enzymol. Relat. Areas Mol. Biol. 40*, 141–238.
Krauspe, R., & Parthier, B. (1974) *Biochem. Physiol. Pflanz. 165*, 18–36.
Lea, P. J., & Norris, R. D. (1977) *Prog. Phytochem. 4*, 121–167.
Miles Biochemical Co. Catalogue (1979) p 103, Miles Laboratories, Inc.
Mirande, M., Kellerman, O., & Waller, J.-P. (1982a) *J. Biol. Chem. 257*, 11049–11055.
Mirande, M., Cirakoglu, B., & Waller, J.-P. (1982b) *J. Biol. Chem. 257*, 11055–11063.
Pan, F., Lee, H. H., Pai, S. H., Yu, T. C., Guoo, J. Y., & Duh, G. M. (1976) *Biochim. Biophys. Acta 452*, 271–283.
Putney, S. D., Royal, N. J., deVegvar, H. N., Herlihy, W. C., Biemann, K., & Schimmel, P. (1981) *Science (Washington, D.C.) 213*, 1497–1500.
Robers, W. K., & Olsen, M. L. (1976) *Biochim. Biophys. Acta 454*, 480–492.
Schneller, J. M., Schneller, C., & Stahl, A. J. C. (1976) in *Genetics and Biogenesis of Chloroplasts and Mitochondria* (Bucher, Th., et al., Eds.) North-Holland Publishing Co., Amsterdam and New York.
Schutter, W. G., Van Bruggen, E. F. J., Bonaventura, J., Bonaventura, C., & Sullivan, B. (1977) in *Structure and Function of Hemocyanin* (Bannister, J. V., Ed.) pp 13–21, Springer-Verlag, Berlin.
Scopes, R. K. (1977) *Biochem. J. 161*, 253–263.
Soll, D., & Schimmel, P. R. (1974) *Enzymes, 3rd Ed. 10*, 489–538.
Swamy, G. S., & Pillay, D. T. N. (1982) *Plant Sci. Lett. 25*, 73–84.
Zaccai, G., Morin P., Jacrot, B., & Moras, D. (1979) *J. Mol. Biol. 129*, 483–500.

Binding of Spin-Labeled Carboxyatractylate to Mitochondrial Adenosine 5′-Diphosphate/Adenosine 5′-Triphosphate Carrier As Studied by Electron Spin Resonance†

Anton Munding,* Klaus Beyer, and Martin Klingenberg

ABSTRACT: The spin-label 2,2,5,5-tetramethyl-1-oxy-3-pyrroline-3-carboxylic acid was attached to the inhibitor carboxyatractylate of the mitochondrial ADP/ATP carrier. Being closely linked to the inhibitor, the spin-label should reflect the mobility of the carboxyatractylate. When bound to the carrier in mitochondria, spin-labeled carboxyatractylate reveals a most unusual hyperfine splitting of 72 G. A second spectral component with a hyperfine splitting of 62 G is also mainly due to carrier-bound inhibitor. A similar spectrum with somewhat reduced hyperfine splitting was observed with the detergent-solubilized protein, whereas reincorporation into phospholipid membranes yielded almost the same spectra as in mitochondria. The carrier-bound spin-label is concluded to be highly immobilized. The less immobilized spectral component is discussed in terms of strongly anisotropic label motion. In addition, the unusual splitting is interpreted to indicate the highly polar environment of the nitroxide. The interpretations are supported by the temperature dependence, which indicates a reversible progressive spin-label mobilization up to 50 °C. Membrane-impermeable reducing agents showed that the spin-label is easily accessible from the aqueous phase.

The ADP/ATP carrier, being the most abundant integral protein of the inner-mitochondrial membrane, should be an interesting example for spin-labeling studies on membrane protein. A highly specific and tightly binding ligand is the inhibitor carboxyatractylate (CAT)[1] (Klingenberg, 1976). A

† From the Institut für Physiologische Chemie, Physikalische Biochemie und Zellbiologie der Universität München, 8000 München 2, Federal Republic of Germany. *Received August 25, 1982.* This work was supported by a grant from the Deutsche Forschungsgemeinschaft (Be 828/2).

[1] Abbreviations: ATR, atractylate; CAT, carboxyatractylate; CATSL, spin-labeled CAT (for structure, see Chart I); ESR, electron spin resonance; EGTA, ethylene glycol bis(β-aminoethyl ether)-*N,N,N′,N′*-tetraacetic acid; EDTA, ethylenediaminetetraacetic acid.

0006-2960/83/0422-1941$01.50/0

suitable label can be introduced into this inhibitor in order to obtain a molecular probe for the mobility of the carrier protein. Also, the environment of the CAT binding site, which is supposed to be identical with the binding center for ADP from the "c" side, can thus be probed.

A label suitable for this approach should be intrinsically rigid and positioned as close as possible to the inhibitor molecule without perturbing the binding behavior of CAT. Conventional ESR is sensitive to motional correlation times in the range of 10^{-10}–10^{-7} s. In addition, it is a sensitive tool for the detection of the polarity of the spin-label environment (Lassmann et al., 1973).

In the present study, the spin-label furnished evidence for the strong immobilization of the inhibitor upon binding to mitochondria. These studies were extended to the Triton-solubilized and membrane-reincorporated protein (Krämer & Klingenberg, 1980), in order to compare the motional properties of the CAT binding site in different states. It thus offers a measure for the tolerance of the protein toward altered environments.

Two earlier papers described the binding of spin-label acyl derivatives of the inhibitors atractylate (ATR) and coenzyme A (Devaux et al., 1975; Lauquin et al., 1977). However, these investigations aimed at the elucidation of the lipid–protein interaction in order to get a structural model of the membrane-bound carrier molecule.

Materials and Methods

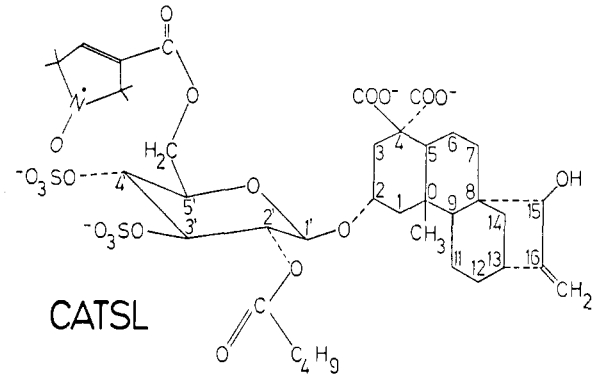
Chemicals. The inhibitors CAT and ATR were obtained from Boehringer-Mannheim (FRG); 2,2,5,5-tetramethyl-1-oxy-3-pyrroline-3-carboxylic acid was purchased from Eastman (Rochester, NY).

Preparation of Spin-Labeled Carboxyatractylate (CATSL). Dimethylformamide was treated with Dowex 50W in the H^+ form in order to remove traces of amine and subsequently thoroughly dried with 4-Å molecular sieves. A total of 100 mg of carbonyldiimidazole was dissolved in 800 μL of this pretreated solvent. This solution was added to 50 mg of 2,2,5,5-tetramethyl-1-oxy-3-pyrroline-3-carboxylic acid. After 10 min at room temperature, a solution of 40 mg of CAT in 800 μL of water was added. After 10 h at room temperature, the mixture was centrifuged. The yellow supernatant was lyophilized. The residue was washed twice with 2 mL of acetone. After removal of the acetone by a stream of nitrogen, the residue was dissolved in 20% ethanol and separated by thin-layer chromatography in chloroform–methanol–water–acetic acid (55:22:4:8 v/v/v/v). CATSL was detected by staining with vanillin reagent at an R_f value of 0.38. The extracted CATSL band gave a single spot on thin-layer chromatography. The yield was about 4% as estimated by ESR (Chart I).

CATSL Binding to Mitochondria. Beef heart mitochondria were isolated essentially as described previously (Blair, 1967) and used immediately or after storage at −176 °C. Within 20 min after addition of CATSL, the ESR signal is decreased by about 50% without changing the line shape. For prevention of reduction of the spin-label during measurement, the mitochondria were preincubated with excess KCN, rotenone, and antimycin. On comparison of the ESR line shape, we have found no difference by the addition of these agents and the use of between freshly prepared and frozen and thawed mitochondria.

For the CATSL binding experiments, beef heart mitochondria (40 mg/mL) in 250 mM sucrose, 10 mM 4-morpholinepropanesulfonic acid, 2 mM $MgCl_2$, and 0.5 mM EGTA, pH 7.2 (buffer M), were loaded with CATSL (as

Chart I



indicated under Results) in the presence of 50 μM ADP. Incubation time was 10 min at 10 °C. The mitochondria were spun down at 12000*g* for 4 min and washed 3 times with the same buffer in order to remove unbound CATSL. For ESR measurements, 180 or 80 μL of the pellet (70 mg/mL) was sucked into an ESR quartz flat cell.

CATSL Binding to Isolated Protein and Reincorporation into Liposomes. The [^{3}H]ATR-loaded adenine nucleotide carrier was isolated in Triton X-100 in 100 mM Na_2SO_4, 10 mM 4-morpholinepropanesulfonic acid, 0.05 mM EDTA, and 0.05 mM NaN_3 at pH 7.2 (buffer P), as described by Aquila & Klingenberg (1982). [^{3}H]ATR could be removed easily by addition of CATSL. The final protein concentration after pressure dialysis was about 6–12 mg/mL. The Triton content was reduced by treatment with 7–14 mg of Bio-Beads SM-3/mg of Triton.

The CATL protein was reincorporated into liposomes of different phospholipid composition [phosphatidylcholine; phosphatidylcholine–phosphatidylethanolamine (60:40 mol/mol); phosphatidylcholine–phosphatidylethanolamine–cardiolipin (55:35:10 mol/mol/mol)] according to Beyer & Klingenberg (1983). The loaded liposomes were spun down at 12000*g* for 3 min, and the pellet was sucked into the ESR flat cell.

Egg phospholipids were prepared as described previously (Wells & Hanahan, 1969; Beyer & Klingenberg, 1978). Protein concentration was determined by a modified biuret (Kröger & Klingenberg, 1966) or Lowry method in the presence of 1% sodium dodecyl sulfate (Helenius & Simons, 1972).

ESR Measurements. ESR spectra were recorded on a JEOL JES-PE-3X spectrometer equipped with a variable-temperature accessory. All spectra were recorded with a modulation frequency of 100 kHz, modulation amplitude of 2 G, and a microwave power of about 50 mW. The sweep width was calibrated from the residual Mn^{2+} in SrO (Bolton et al., 1972).

Results

Binding of Spin-Labeled CAT to Mitochondria. Beef heart mitochondria pretreated with inhibitors of mitochondrial respiration were incubated with CATSL, and excess CATSL was removed as described under Materials and Methods. At 10 °C the ESR spectrum of the pelleted mitochondria shows a line shape indicating strong immobilization of the CATSL (Figure 1A). The hyperfine separation of the outer narrow signals is 71.4 G (Figure 1A), which indicates extremely slow label motion. Figure 1A shows the presence of a second, more weakly immobilized component. The low-field peaks of these components are denoted by S and W, respectively, in Figure 1A. Quantitation of the S component in terms of a rotational

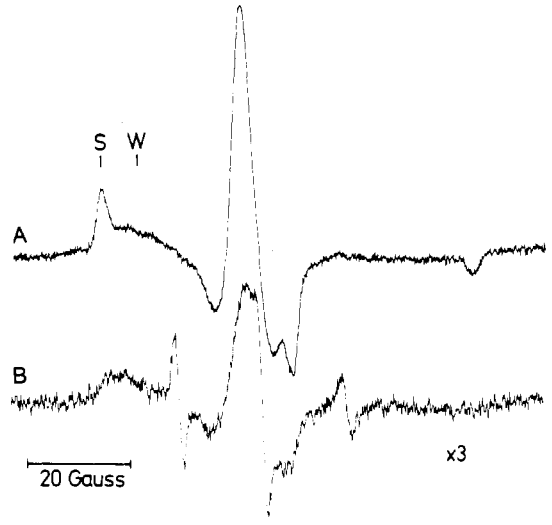


FIGURE 1: ESR spectra after addition of CATSL to mitochondria (A) and to mitochondria preloaded with 16 nmol of CAT/mg of protein (B). 50 μM ADP was added with CATSL in (A) and with CAT in (B). Mitochondria were incubated with 2 nmol of CATSL/mg of protein for 10 min. Unbound CATSL was removed by repeated washing with mitochondrial buffer M. Spectra were recorded at 10 °C.

correlation time τ_c is complicated for the following reasons. First, the extreme hyperfine separation of 71.4 G and the narrow line width of the S peak signify that the spin-label motion is beyond the sensitivity limit of conventional ESR of 10^{-7} s. Second, in particular at elevated temperatures (see below), the interference of the W component hampers the correct determination of the hyperfine separation.

So that one could discern the contribution of the specific binding to the spectrum, mitochondria were preincubated with excess CAT before CATSL addition. The spectrum obtained after removal of excess spin-label is shown in Figure 1B. The line shape is characteristic of weak spin-label immobilization and of a trace of free tumbling nitroxide. The strongly immobilized component is absent, indicating that the corresponding binding site is occupied by CAT. Conversely, addition of 10-fold excess CAT to CATSL-preloaded mitochondria released about 15% CATSL in 15 min at 10 °C.

Titration of the mitochondria with CATSL results in increasing signal heights, s and w, corresponding to the components S and W, as shown in Figure 2A. At about 1.2 μmol of CATSL/g of protein a break appears that separates high-affinity from apparently low-affinity sites. Below 0.8 μmol of CATSL/g of protein no unbound spin-label could be observed in the supernatants. The parallel increase of the S and W portions indicates that both reflect binding to similar sites.

Determination of the binding constant of CATSL is difficult due to the presence of these superimposed spectra. The question was investigated whether the two portions show a different sensitivity to CAT. Mitochondria were preincubated with CAT at a concentration of up to 20 μmol of CAT/g of protein, followed by addition of 1.6 μmol of CATSL/g of protein (Figure 2B). The amplitudes s and w decrease linearly. Extrapolation of the s curve to zero amplitude shows that about 1.5 μmol of CAT/g of protein is necessary to suppress the S spectral component. At about the same CAT concentration, the signal height of the W component levels off to a nearly constant value w'. Below a CAT concentration of 1.5 μmol/g of protein, the ratio $s/(w - w')$ is almost constant. This agrees with a value of about 1.6 μmol/g of protein determined for CAT binding in mitochondria (Klingenberg et al., 1975) and identified exclusively as ADP/ATP carrier sites. The equal

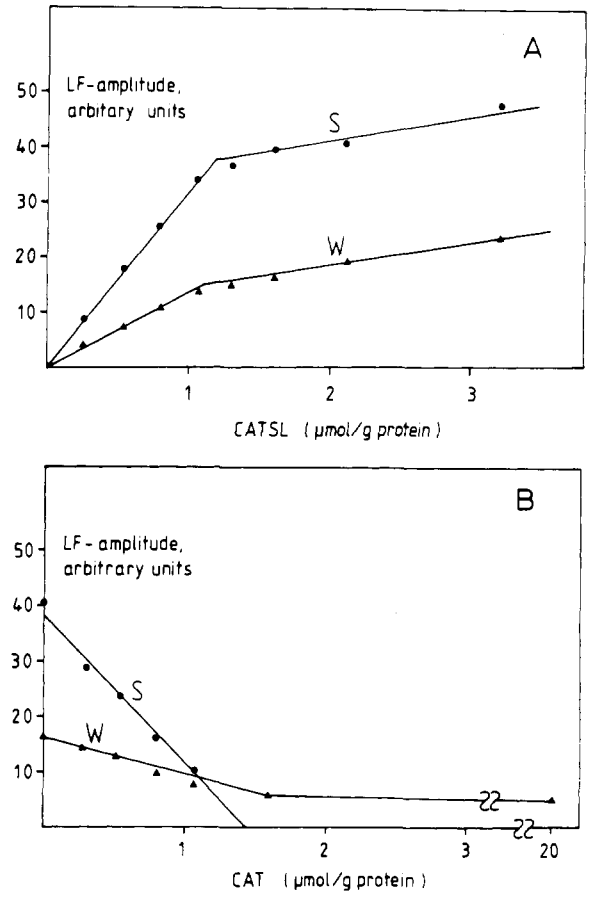


FIGURE 2: Amplitudes of low-field (LF) peaks S and W (see Figure 1) of bound CATSL upon titration of mitochondria with CATSL or CAT. (A) Titration of untreated mitochondria with CATSL. (B) Preincubation for 15 min with increasing amounts of CAT, followed by addition of 1.6 nmol of CATSL/mg of protein. All spectra were recorded at 10 °C, same incubation conditions as in Figure 1.

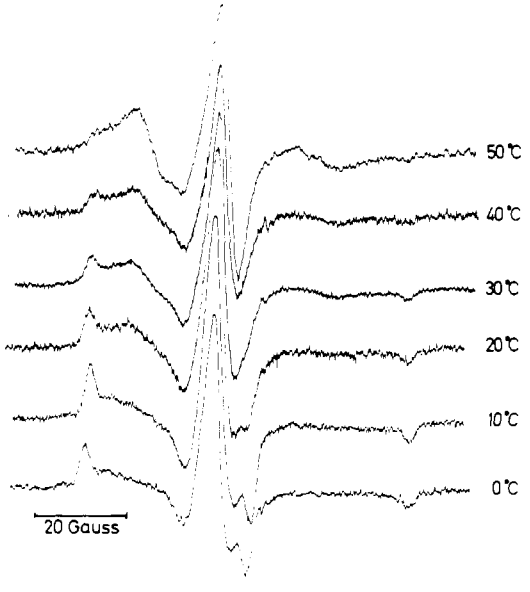


FIGURE 3: Temperature dependence of ESR spectra of CATSL bound to mitochondria. Same conditions as in Figure 1A.

sensitivity of s and $w - w'$ to CAT indicates the specific binding of both components to the carrier. W' seems to originate from different sites.

For clarification of the origin of the different spectral components, attempts were made to influence the ratio s/w. The strong temperature dependence of the ESR spectra is

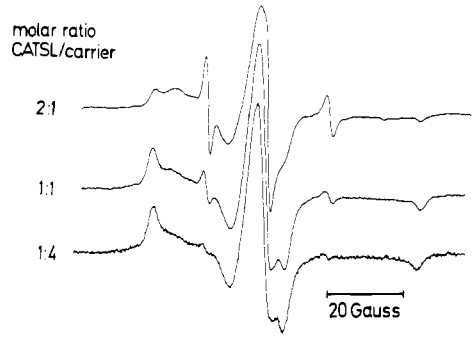


FIGURE 4: ESR spectra of CATSL bound to Triton-solubilized ADP/ATP carrier at different molar ratios of CATSL/carrier. Spectra were recorded at 2 °C. Concentration of protein was 5.8 mg/mL and of Triton 95 mg/mL.

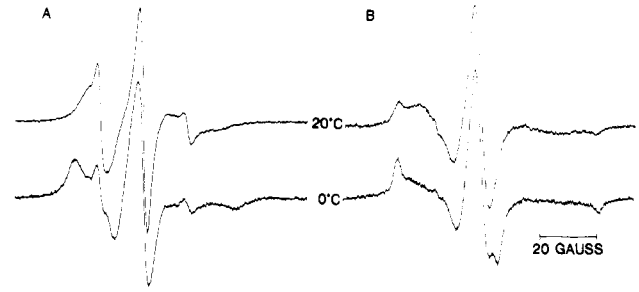


FIGURE 5: Temperature dependence of ESR spectra of 0.05 mM CATSL in buffer P containing 104 mg of Triton X-100/mL (A) and of CATSL bound to Triton-solubilized protein (B). Molar ratios of CATSL/carrier = 0.55:1; concentration of protein was 5.6 mg/mL and of Triton 104 mg/mL.

shown in Figure 3. On heating the sample from −20 to 50 °C, the S peak apparently decreased in favor of the W component. Above 50 °C, released CATSL gradually appears (not shown). The label once released remains unbound at lower temperatures, presumably as a consequence of protein denaturation. The hyperfine separation $2A_{zz}$ of the S component decreases from 72.4 G at −20 °C to 69.8 G at 30 °C, indicating greater label mobility (cf. Figure 7).

The unusually large hyperfine splitting of 71.7 G at −5 °C cannot be explained solely in terms of spin-label immobilization. Formation of a hydrogen bond between the nitroxide and a proton donor in the binding site may substantially contribute to the enhanced splitting. This effect has been demonstrated earlier with covalently spin-labeled hemoglobin (Johnson, 1981) and spin-labeled hemocyanin bound to an antibody (Humphries & McConnell, 1976). However, 8 M urea at 10 °C did not affect the overall line shape of the ESR spectrum. Even after 1 h at 10 °C, not more than 20% of the CATSL was liberated, the bond portion being unaffected. The location of the nitroxide moiety was probed by impermeant hydrophilic reducing agents. Fe^{2+} or ascorbate led to rapid and complete reduction of bound CATSL. Thus the nitroxide should be easily accessible from the aqueous phase. Obviously, the overall conformation of the protein as well as the environment of the nitroxide group is relatively insensitive to urea.

The spin-labeled CAT derivative carrying four negative charges can be assumed to bind tightly to the carrier protein by strong ionic forces. High ionic strength in the medium should weaken this interaction. However, addition of up to 2 M NaCl did not change either binding or line shape. Thus hydrophobic interactions seem to contribute essentially to the tight inhibitor binding.

The spin-label derivative of ATR, which lacks one of the two geminal carboxylic groups at CAT, yielded on binding to mitochondria almost the same ESR spectrum as CATSL. Also the ratio of the two components S and W was the same. Merely a small reduction in the hyperfine splitting of the S component was observed (not shown).

Binding to Solubilized and Reincorporated Carrier Protein. The isolated ADP/ATP carrier can be obtained in stable form as ATR–protein complex by solubilizing the mitochondrial membrane loaded with ATR in Triton X-100, followed by several purification steps (Riccio et al., 1975; Aquila & Klingenberg, 1982). Exchange of bound ATR for CATSL is easily possible due to the tighter binding of the latter.

The ESR spectra of the CATSL-labeled solubilized protein resemble those of labeled mitochondria as long as the molar ratio of CATSL to carrier protein is below 0.6. The superposition of two differently immobilized components is also perceptible in this case (see Figure 4, bottom trace). Minor differences were found in splitting and line width of the outer hyperfine extrema, the splitting being reduced by about 2 G at 0 °C. At larger CATSL/carrier ratios, the signal equivalent to the W component increases (see Figure 4, top spectra).

In order to analyze the W component, we compared the spectra of CATSL bound to the solubilized protein (Figure 5B) with those obtained of CATSL added to a Triton solution without protein (Figure 5A). A two-component ESR spectrum was observed, which indicates weak immobilization of part of the CATSL. The weakly immobilized component probably originates from binding to Triton micelles, since it increases with Triton concentration (not shown). The hyperfine splitting of the weakly immobilized component decreases with increasing temperature. At 20 °C, the Triton-immobilized component exhibits a narrower hyperfine splitting than the W component of the spectra of the CATSL-labeled solubilized protein (see Figure 5). This indicates that in the presence of protein the W component originates mainly from protein-bound spin-label.

So that one could distinguish the contribution of CATSL bound to Triton micelles, most of the free Triton was removed by Bio-Beads (Holloway, 1973). The resulting spectra exhibited the shape of the 1:4 CATSL/carrier spectrum in Figure 4 (bottom trace), irrespective of whether the original spectrum was derived with excess or stoichiometric amounts of CATSL. This shows that both the micellar and protein-bound CATSL contribute to the W signal.

Reincorporation of the inhibitor-labeled carrier in undenatured form into phospholipid liposomes is possible due to the very hydrophobic nature of this protein (Beyer & Klingenberg, 1983; Krämer & Klingenberg, 1980). The solubilized CATSL protein was incorporated into liposomes of varying phospholipid composition (see Materials and Methods). ESR in these preparations revealed a picture very similar to what has been found in mitochondria (Figure 6). Again, two states of restricted label motion are discernible. Spectra recorded at 0, 15, and 30 °C were almost identical with mitochondrial spectra at the same temperatures, irrespective of the phospholipid composition of the protein-loaded liposomes.

Discrimination between the various bound components by a titration experiment with CAT as depicted in Figure 2 is not feasible in this case due to the presence of multilamellar membranes that are impermeable to CAT. Addition of CATSL to a dispersion of pure egg lecithin revealed some CATSL binding to the phospholipids (Figure 6B).

Hyperfine Splitting in Frozen Solutions. The unusually large hyperfine separation found for the carrier-bound CATSL in mitochondria and also for the isolated protein at 0 °C is due to the polarity of the environment of the NO group. For determination of the influence of solvent on this polarity, ESR

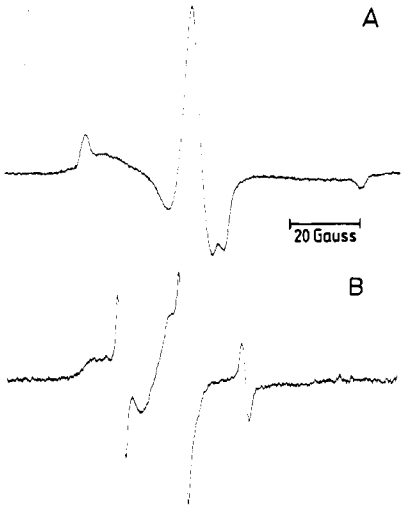


FIGURE 6: CATSL-labeled carrier protein reincorporated into phosphatidylcholine liposomes (A). Addition of CATSL to a dispersion of phosphatidylcholine (B). Spectra of the pellets recorded at 10 °C.

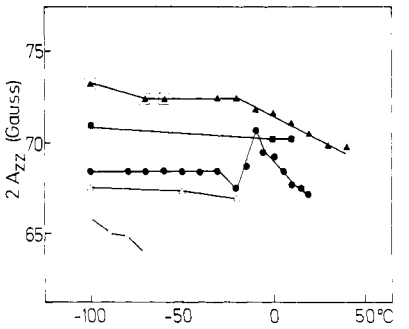


FIGURE 7: Low-temperature behavior of hyperfine splitting $2A_{zz}$ of carrier-bound and free CATSL in various solvents. CATSL bound to mitochondria (▲); same sample lyophilized (■); CATSL bound to solubilized protein (●); free CATSL in buffers M and P (□), in 100 mM Triton X-100 (△), and in ethanol (○). Splittings in M and P were identical.

spectra of carrier-bound and free CATSL in different frozen solutions were compared. At −100 °C, almost all contributions from molecular motions to the ESR spectra are frozen out. The temperature dependence of the $2A_{zz}$ value is shown in Figure 7. The largest hyperfine separation of 73.4 G was found for the mitochondrial suspension and frozen buffer solutions without Triton. Lyophilization of the labeled mitochondria resulted in a lowered separation of 70.9 G. The splitting for the solubilized CATSL protein and the unbound CATSL in protein buffer with a comparable Triton concentration of about 15% was 68.4 G below −20 °C. Upon melting of the frozen protein-containing solution, the hyperfine separation unexpectedly increased from about 68 to 71 G. Rearrangement of the detergent molecules may be responsible (Beyer, 1982), whereas micellar rotation may explain the continuous decrease in hyperfine splitting on further increase of the temperature. The $2A_{zz}$ value for CATSL in frozen ethanol and H_2O at −100 °C is 65.8 and 71.3 G, respectively.

The hyperfine separations for the CATSL precursor 2,2,5,5-tetramethyl-1-oxy-3-pyrroline-3-carboxylic acid in frozen protein buffer, ethanol, and 36% Triton were consistently about 2 G larger than those obtained with CATSL (not shown in Figure 7).

Discussion

CATSL Binding and Protein Mobility. The inhibitor CAT binds to the ADP/ATP carrier with high affinity and specificity only from the c side of the inner-mitochondrial membrane. Obviously, the attachment of the spin-labeled pyrrolinecarboxylic acid to the primary alcoholic group of the sugar moiety of CAT does only slightly interfere with this interaction. The nitroxide close to the ring system of the glycoside should sense the overall motion of the inhibitor molecule. In contrast, earlier ESR investigations on the ADP/ATP carrier employed spin-labels attached to a lipid spacer (Devaux et al., 1975; Lauquin et al., 1977). A questionable structural model was deduced. However, no information about the protein mobility and the properties of the binding site was obtained.

The mobility of membrane-bound proteins has been determined mostly by ESR (Marsh, 1981) and by flash-induced absorption anisotropy (Cherry, 1978) of covalently attached labels.

For the purified cyctochrome *c* reductase and oxidase reconstituted into extracted mitochondrial lipids, a rotational correlation time τ_c of 70 and 40 μs, respectively, was determined by saturation-transfer ESR (Swanson et al., 1980; Quintanilha et al., 1982). Thomas & Hidalgo (1978) and Andersen et al. (1981) estimated an effective rotational correlation time of about 50 μs for the maleimide spin-labeled Ca^{2+}-ATPase in sarcoplasmatic reticulum.

The rotational diffusion of the ADP/ATP carrier in submitochondrial particles was investigated by observation of flash-induced absorption anisotropy (Müller et al., 1982). A time constant for the rotation of the translocator of 240 μs at 5 °C and 100 μs at 37 °C was found.

The common feature in all experiments with bound CATSL is the superposition of several ESR signals, reflecting different degrees of immobilization. The prevailing component, denoted by S in Figure 1A, exhibits a very strong label immobilization. The large hyperfine separation of 71.0 G at 10 °C indicates that the reorientation rate of the label is $<10^7$ s^{-1}, which is below the sensitivity limit of conventional ESR. Also, the relatively narrow line width of the outer peaks shows that the mobility of the protein in mitochondria is well below this limit (Mason et al., 1974). Of course, protein may have a residual tumbling motion beyond this range. To our knowledge, a comparable immobilization of spin-labeled protein ligands has never been observed.

The origin of the less immobilized component, denoted by W, reflecting a reorientation rate of about 10^8 s^{-1}, is difficult to interpret. Preloading mitochondria with CAT suppresses the S component completely, whereas the W component contains a CAT-insensitive portion W′, which amounts to about 30% of the W signal. It is difficult to give an unambiguous interpretation of the CAT-sensitive contribution to W, which we attribute to CATSL bound also to carrier sites. The same signal component is seen with the purified solubilized carrier. Three tentative explanations may be given. (1) The spin probe may undergo librational motion, independent of the protein and inhibitor moiety (Johnson, 1978). Such spin-label wagging would essentially modulate the slow reorientation brought about by the protein reorientation. Theoretical line-shape calculations for anisotropic spin-label reorientations have revealed spectra similar to those obtained in the present work (Mason et al., 1974). Thus the apparent superposition of two spectra may originate from the anisotropic nature of the CATSL motion. Increasing temperature would lead to more isotropic spin-label motion in agreement with the experimentally observed changes in line shape. (2) The carrier may exist in two CAT binding states, c and c′, respectively, resulting in different inhibitor immobilization. At least two possibilities can be envisaged. The two types of binding are in equilibrium, or there are two separate populations of carrier

protein. In the latter case, one population may be due to a partial damage of the protein caused by the preparation procedure. (3) Each carrier molecule contains strongly and weakly immobilized sites, analogous to high- and low-affinity sites.

A strict discrimination between these models is impossible at present. In choosing between these models, one has to consider that the ratio of $s/(w - w')$ is constant during the titration (cf. Figure 2) and also in the isolated and reincorporated system (cf. Figures 6 and 7). This argues against a heterogeneity by preparative damage. The reproducibility of this signal ratio in many preparations and also the reversibility of the temperature changes up to 50 °C excludes an artificial damage interpretation as a source of the W component. The observation that the addition of excess CAT to CATSL-labeled mitochondria results in a parallel decrease of the two components makes the two-site model unlikely. Thus we prefer an interpretation in terms of an anisotropic spin-label motion.

Polarity Effects. From the known crystal data for the hyperfine tensor of the compound 2,2,5,5-tetramethyl-3-carbamido-3-pyrroline-1-oxy, a rigid limit value of $2A_{zz}$ for the hyperfine splitting of about 62 G can be expected (Capiomont et al., 1974). It has been shown previously that a polar environment enhances the nitroxide hyperfine separation (Lassmann et al., 1973; Humphries & McConnell, 1976). A qualitative similar effect has been reported upon formation of an H bond in spin-labeled hemoglobin. In this case, a strong increase in hyperfine splitting at low temperature was found (Johnson, 1981). This has been attributed to the shift in hydrogen-bond equilibrium. Thus in the present system three possible causes for the large hyperfine separation must be considered. First, the nitroxide group may be located in a rather polar environment, presumable hydrogen bonded to the surrounding solvent. Hydrogen bonds may exist either between the nitroxide and the protein or within the CATSL molecule. Spectra of frozen samples of protein-bound and free CATSL were recorded, in order to decide between these possibilities. At −100 °C, almost all molecular motion is absent. Thus the considerable variations in hyperfine splitting observed in different samples can be attributed to the different environments of the nitroxide. The splitting for CATSL bound to the carrier either in mitochondria or in the solubilized form resembles closely that found for free CATSL in mitochondrial buffer or in Triton, respectively. This suggests that in the frozen solutions the hyperfine separation is determined by the surrounding bulk solvent rather than by a special inter- or intramolecular interaction. Freezing of CATSL in ethanol gives a much narrower splitting in agreement with the more hydrophobic nature of this solvent. Lyophilization of CATSL-labeled mitochondria reduces the splitting but does not lead to the crystal values, indicating that still some water is present.

The temperature dependence of the hyperfine separation of the Triton-solubilized CATSL protein exhibits an unexpected behavior. Slowing down of micelle rotation by lowering the temperature from 30 to −10 °C may explain that the $2A_{zz}$ value in the solubilized protein approaches that in the mitochondrial system. Upon freezing of the solvent, a dramatic reduction in hyperfine separation occurs. It has been shown recently that a micellar solution of Triton upon freezing undergoes a phase transition, presumably forming a lamellar structure (Beyer, 1982). In this state, competition of water binding to the protein and to the oxyethylene groups of the detergent may lead to a dehydration of the protein by the hydrophilic tails of the detergent.

The observation that the bound spin-label reacts very rapidly with reducing agents further supports the conclusion that the nitroxide group of protein-bound CATSL is located in the aqueous phase. The observation that urea has only little influence upon the large hyperfine splitting indicates that the nitroxide is strongly hydrated. A hydrogen bond to a donor group in the protein molecule or intramolecular hydrogen bonding should be disrupted by the addition of urea, leading to a decrease in the hyperfine splitting. Inspection of a space-filling model, assuming that the four negative charges of CAT are directed toward the protein surface, reveals that the nitroxide moiety is turned away from the binding site, presumably extending to the aqueous environment.

Conclusions

The detailed analysis of the unusual ESR spectra led to the following results. The spectral component S exhibiting very large hyperfine splitting should reflect, at least at low temperatures, the rotation rate of the carrier protein in the membrane. Due to the limitation of conventional ESR, it can only be inferred that this rotation rate is far below 10^7 s^{-1}, whereas the rate for the solubilized protein is in the range of 10^7 s^{-1}. The second spectral component (W) most likely originates from very anisotropic motion of the spin-labeled inhibitor.

Acknowledgments

We thank Dr. D. Marsh for helpful discussions. The excellent technical assistance of Irmgard Greimel and Renate Lafuntal is gratefully acknowledged.

Registry No. CATSL, 84602-22-2; ADP/ATP carrier, 9068-80-8.

References

Andersen, J. P., Fellmann, P., Møller, J. V., & Devaux, P. F. (1981) *Biochemistry 20*, 4928–4936.

Aquila, H., & Klingenberg, M. (1982) *Eur. J. Biochem. 122*, 141–145.

Beyer, K. (1982) *J. Colloid Interface Sci. 86*, 73–89.

Beyer, K., & Klingenberg, M. (1978) *Biochemistry 17*, 1424–1431.

Beyer, K., & Klingenberg, M. (1983) *Biochemistry 22*, 639–645.

Blair, P. V. (1967) *Methods Enzymol. 10*, 78–81.

Bolton, J. R., Borg, D. C., & Swartz, H. M. (1972) in *Biological Applications of Electron Spin Resonance* (Swartz, H. M., Bolton, J. R., & Borg, D. C., Eds.) pp 63–118, Wiley-Interscience, New York.

Capiomont, A., Chion, B., Lajzerowicz, J., & Lemaire, H. (1974) *J. Chem. Phys. 60*, 2530–2535.

Cherry, R. J. (1978) *Methods Enzymol. 54*, 47–61.

Devaux, P. F., Bienvenue, A., Lauquin, G., Brisson, A. D., Vignais, P. M., & Vignais, P. V. (1975) *Biochemistry 14*, 1272–1280.

Helenius, A., & Simons, K. (1972) *J. Biol. Chem. 247*, 3656–3661.

Holloway, P. M. (1973) *Anal. Biochem. 53*, 304–308.

Humphries, G. M. K., & McConnell, H. M. (1976) *Biophys. J. 16*, 275–277.

Johnson, M. E. (1978) *Biochemistry 17*, 1223–1228.

Johnson, M. E. (1981) *Biochemistry 20*, 3319–3328.

Klingenberg, M. (1976) in *The Enzymes of Biological Membranes* (Martonosi, A., Ed.) Vol. 3, pp 383–438, Plenum, New York.

Klingenberg, M., Grebe, K., & Scherer, B. (1975) *Eur. J. Biochem. 52*, 351–363.

Krämer, R., & Klingenberg, M. (1980) *Biochemistry 19*, 556–560.
Kröger, A., & Klingenberg, M. (1966) *Biochem. Z. 344*, 317–336.
Lassmann, G., Ebert, B., Kuznetsov, A. N., & Damerau, W. (1973) *Biochim. Biophys. Acta 310*, 298–304.
Lauquin, G. J. M., Devaux, P. F., Bienvenue, A., Villiers, C., & Vignais, P. V. (1977) *Biochemistry 16*, 1202–1208.
Marsh, D. (1981) in *Membrane Spectroscopy* (Grell, E., Ed.) pp 51–142, Springer Verlag, Berlin and Heidelberg.
Mason, R. P., Polnaszek, C. F., & Freed, J. H. (1974) *J. Phys. Chem. 78*, 1324–1329.
Müller, M., Krebs, J. J. R., Cherry, R. J., & Kawato, S. (1982) *J. Biol. Chem. 257*, 1117–1120.
Quintanilha, A. T., Thomas, D. D., & Swanson, M. (1982) *Biophys. J. 37*, 68–69.
Riccio, P., Aquila, H., & Klingenberg, M. (1975) *FEBS Lett. 56*, 133–138.
Swanson, M. S., Quintanilja, A. T., & Thomas, D. D. (1980) *J. Biol. Chem. 255*, 7494–7502.
Thomas, D. D., & Hidalgo, C. (1978) *Proc. Natl. Acad. Sci. U.S.A. 75*, 5488–5492.
Wells, M. A., & Hanahan, D. J. (1969) *Methods Enzymol. 14*, 179–183.

Changes in Chemical Properties of Mitochondrial Adenosinetriphosphatase upon Removal of Tightly Bound Nucleotides†

James K. Tamura and Jui H. Wang*

ABSTRACT: The removal of tightly bound nucleotides from mitochondrial F_1-ATPase was found to affect the inhibition by ADP and chemical reactivity toward 7-chloro-4-nitro-2,1,3-benzoxadiazole (NBD-Cl) and sulfhydryl reagents. Preincubation of nucleotide-depleted F_1 with 40 μM ADP in the presence of ethylenediaminetetraacetic acid (EDTA) resulted in a 51% inhibition of the steady-state level of ATPase activity whereas only a 25% inhibition was observed for native F_1. Both partially inhibited states of the enzyme could be reversed by the subsequent addition of ATP. Measurement of [^{14}C]ADP binding to nucleotide-depleted F_1 in the presence of EDTA reveals three equivalent ADP binding sites with a K_d of 0.45 μM, and a fourth site of lower affinity. The sulfhydryl reagents 5,5′-dithiobis(2-nitrobenzoic acid) (DTNB) and *N*-ethylmaleimide (NEM) were found to inhibit the ATPase activity of nucleotide-depleted F_1 but not native F_1 or nucleotide-depleted F_1 in the presence of ADP or ATP. Polyacrylamide gel electrophoresis of nucleotide-depleted F_1 labeled with [^{14}C]NEM gave a 2-fold increase in incorporation into the ($\alpha + \beta$) subunits and a 7-fold increase in label in the γ subunit after 90 min compared to when ADP was present during the reaction. ADP binding to the noncatalytic sites enhanced the rate of inhibition of nucleotide-depleted F_1 by NBD-Cl about 2-fold while retarding the subsequent intramolecular transfer from an essential phenol group to an amino group about 2.8-fold. The results suggest a conformational change in F_1 caused by changes in nucleotide–protein interaction at the noncatalytic sites.

The mitochondrial F_1-ATPase[1] carries the catalytic sites for the synthesis of ATP from ADP and P_i during oxidative phosphorylation. The soluble F_1 portion is composed of five different subunit components (Knowles & Penefsky, 1972a,b), designated α, β, γ, δ, and ϵ. The covalent labeling by photoaffinity analogues of ATP (Wagenvoord et al., 1977; Scheurich et al., 1978; Lunardi & Vignais, 1979; Williams & Coleman, 1982) and phosphate (Lauquin et al., 1980) as well as by alkylating or acylating ATP analogues (Budker et al., 1977; Esch & Allison, 1978; Drusta et al., 1979) has led to the suggestion that the β subunit carries the catalytic site.

Also present on F_1 from mitochondria (Harris et al., 1977; Hashimoto et al., 1981), chloroplasts (Harris & Slater, 1975; Bruist & Hammes, 1981), and bacterial systems (Maeda et al., 1976; Ohta et al., 1980a) are noncatalytic nucleotide binding sites thought to function in regulation. These latter sites have a much higher degree of specificity for adenine nucleotides than the catalytic sites (Harris et al., 1978; Recktenwald & Hess, 1980). Chemical affinity labeling experiments (Kozlov & Milgrom, 1980) in addition to nucleotide binding studies using isolated subunits of *Escherichia coli* (Dunn & Futai, 1980) and thermophilic bacteria (Ohta et al., 1980a) have led to the suggestion that the noncatalytic nucleotide-binding sites are on the α subunits. Nucleotides bound to the noncatalytic sites are empirically termed as "tightly bound" or "nonexchangeable".

More detailed information on the physical and functional relationships between or within the two classes of nucleotide binding sites may be essential to a clear understanding of the mechanisms of synthesis and utilization of ATP by F_1. Cooperativity between multiple hydrolytic sites on isolated F_1 has been demonstrated by Grubmeyer & Penefsky (1981) and Nalin & Cross (1982). In addition, measurements of the rates of the hydrogen exchange on isolated α and β subunits from thermophilic bacteria have provided evidence that the α sub-

† From the Bioenergetics Laboratory, State University of New York, Buffalo, New York 14214. *Received August 18, 1982.* This work was supported in part by research grants from the National Science Foundation (PCM 8117952) and the National Institute of General Medical Sciences (GM 19990).

[1] Abbreviations: AMP-PNP, 5′-adenylyl imidodiphosphate; DTNB, 5,5′-dithiobis(2-nitrobenzoic acid); DTT, dithiothreitol; EDTA, ethylenediaminetetraacetic acid; F_1, mitochondrial coupling factor 1; Hepes, *N*-(2-hydroxyethyl)piperazine-*N*′-2-ethanesulfonic acid; Hepes–EDTA–glycerol buffer, 50 mM Hepes (pH 7.5), 2 mM EDTA, 25 mM NaCl, and 25% glycerol; NBD-Cl, 7-chloro-4-nitro-2,1,3-benzoxadiazole (also named 4-chloro-7-nitrobenzofurazan); NBD-F_1, F_1 labeled by NBD-Cl; nd-F_1, nucleotide-depleted F_1; NEM, *N*-ethylmaleimide; ATPase, adenosinetriphosphatase.

0006-2960/83/0422-1947$01.50/0