Meissner, G. (1986) *Biochemistry 25*, 244-251.
Michalak, M., Famulski, K., & Carafoli, E. (1984) *J. Biol. Chem. 259*, 15540-15547.
Niggli, V., Adunyah, E. S., & Carafoli, E. (1981) *J. Biol. Chem. 256*, 8588-8592.
Repasky, E. A., Pollina, C. M., Menold, M. M., & Hudecki, M. S. (1986) *Proc. Natl. Acad. Sci. U.S.A. 83*, 802-806.
Scales, D., Sabbadini, R., & Inesi, G. (1977) *Biochim. Biophys. Acta 465*, 535-549.
Scharff, O. (1979) *Anal. Chim. Acta 109*, 291-305.
Scharff, O. (1981) *Cell Calcium 2*, 1-27.
Stryer, L., & Bourne, H. R. (1986) *Annu. Rev. Cell Biol. 2*, 391-419.
Sugita, H., Kimura, M., Tarumoto, Y., Tamai, M., Hanada, K., Ishiura, S., Nonaka, I., Ohzeki, M., & Imahori, K. (1982) *Muscle Nerve 5*, 738-744.
Suzuki, K. (1987) *Trends Biochem. Sci. (Pers. Ed.) 12*, 103-105.
Thacore, H. R., Kibler, P. K., Hudecki, M. S., Galindo, J., Jr., Davis, F. B., Davis, P. J., Pollina, C. M., Blas, S. D., & Schoenl, M. (1988) *Biochem. Biophys. Res. Commun. 151*, 1434-1440.
Tovar, A. M., & Verjovski-Almeida, S. (1983) *Mol. Cell. Biochem. 56*, 39-48.
Verjovski-Almeida, S., & Inesi, G. (1979) *Biochim. Biophys. Acta 558*, 119-125.

Molecular Mechanisms Responsible for the Drug-Induced Posttranscriptional Modulation of Ribonucleotide Reductase Levels in a Hydroxyurea-Resistant Mouse L Cell Line†

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ABSTRACT: Ribonucleotide reductase, which catalyzes the formation of deoxyribonucleotides from ribonucleoside diphosphate precursors, is the rate-limiting enzyme in DNA synthesis. The enzyme consists of two nonidentical subunits called M1 and M2, both of which are required for activity. Hydroxyurea, a specific inhibitor of DNA synthesis, acts by destroying the unique tyrosyl free radical of protein M2. Previously, we have described a mouse L cell line which exhibited a stable resistance to high concentrations of hydroxyurea. This mutant cell line contains elevated quantities of both proteins M1 and M2 as a result of corresponding increases in the levels of mRNAs for both subunits. Interestingly, both M1 and M2 protein levels were further elevated when mutant cells were cultured in the presence of hydroxyurea, and this elevation was not accompanied by increases in their corresponding mRNAs. These results indicated that hydroxyurea can modulate ribonucleotide reductase expression posttranscriptionally. In this report, we show that the level of both subunits of ribonucleotide reductase responds to hydroxyurea in a drug concentration dependent manner. Furthermore, results from kinetic studies indicate that protein M2 levels rise much more rapidly than protein M1. Pulse–chase experiments indicated that the half-lives of both the M1 and M2 polypeptides are increased by approximately 2-fold when the mutant cells are cultured in the presence of hydroxyurea. We also present evidence indicating that exposure of these cells to hydroxyurea leads to a relatively slow but specific increase in the rate of biosynthesis of both proteins M1 and M2, as assayed by pulse labeling. Therefore, we conclude that both components of ribonucleotide reductase are synthesized at an increased rate and turn over at a slower rate when these mutant cells are grown in the presence of hydroxyurea. In addition, experiments were performed to examine the effects of exogenously added iron on the biosynthesis of proteins M1 and M2. Interestingly, the results suggested a role for iron in regulating the level of M2 protein when cells are cultured in hydroxyurea-supplemented medium.

Mammalian ribonucleotide reductase is a highly regulated enzyme that is responsible for the conversion of ribonucleotides to their corresponding deoxyribonucleotides, the precursors of DNA synthesis (Thelander & Reichard, 1979; Wright, 1988). This reaction is a rate-limiting step in DNA synthesis, and, therefore, the enzyme plays an important role in the regulation of cell division. In mammalian cells, the enzyme consists of two nonidentical subunits called M1 and M2, both of which have been purified to homogeneity (Thelander et al., 1980, 1985). The M1 protein is a dimer of molecular weight 170 000, and it contains the binding sites for nucleoside triphosphates which act as allosteric effectors (Thelander et al., 1980). The M2 protein is a dimer of molecular weight 88 000, and it contains stoichiometric amounts of a non-heme iron center and a tyrosyl free radical essential for activity (Thelander et al., 1985). The two subunits are differentially regulated during the cell cycle with reductase activity being controlled by the S-phase-dependent synthesis of protein M2

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(Eriksson et al., 1984; Engstrom et al., 1985).

The antitumor agent hydroxyurea enters cells by a diffusion process (Tagger et al., 1987) and specifically inhibits DNA synthesis by inactivating the tyrosyl free radical component of the M2 subunit of ribonucleotide reductase (Graslund et al., 1982). Hydroxyurea has been useful as a selective agent in cell culture for the isolation of drug-resistant cell lines (Wright, 1988; Wright et al., 1988). Mutant cells selected for resistance to hydroxyurea have been shown to overproduce ribonucleotide reductase activity mainly because of an overproduction of the M2 subunit, usually as a direct result of increased M2 mRNA levels and an amplification of the M2 gene (Thelander & Berg, 1986; Wright et al., 1987; McClarty et al., 1987). In addition to increases in protein M2, cell lines selected for resistance to high concentrations of hydroxyurea commonly show elevated protein M1 levels as well (McClarty et al., 1986a; Cocking et al., 1987). These M1-overproducing cells have an accompanying increase in M1 mRNA levels (Thelander & Berg, 1986; Wright et al., 1988; McClarty et al., 1987) and occasionally an amplification of the M1 gene (Cocking et al., 1987; Wright et al., 1988).

In the past, we have characterized a cell line that exhibited a stable resistance to high concentrations of hydroxyurea (McClarty et al., 1986a,b). This cell line contained an elevation in ribonucleotide reductase activity as a result of a 2–3-fold increase in protein M1 levels and a 40–50-fold elevation in protein M2 as compared to wild-type cells (McClarty et al., 1987). The increases in proteins M1 and M2 were accompanied by a corresponding increase in the level of mRNAs for both subunits. Furthermore, the increase in both M1 and M2 mRNAs was a result of an increase in the rate of transcription of the two genes. There was a 6-fold amplification in the gene copy number for M2 but no increase in gene copy number for protein M1. Most interestingly, this mutant cell line displayed a further increase in ribonucleotide reductase activity when the resistant cells were cultured in the presence of hydroxyurea (McClarty et al., 1986a). This hydroxyurea-induced increase in ribonucleotide reductase activity was a result of a further elevation in both protein M1 and M2 levels when the mutant cells were cultured in the presence of drug (McClarty et al., 1987). Surprisingly, this hydroxyurea-induced elevation in both proteins was not accompanied by increases in the levels of the corresponding mRNAs. These results lead us to suggest that hydroxyurea can modulate ribonucleotide reductase expression posttranscriptionally in this mutant cell line. The major objective of the experiments presented in this report is to provide additional information on the mechanism by which ribonucleotide reductase activity is increased in this mutant cell line when it is exposed to hydroxyurea. Our results indicate that hydroxyurea can affect both the half-life and the rate of biosynthesis of proteins M1 and M2.

Materials and Methods

Cell Lines and Culture Conditions. The mutant cell line (SC2) used in this study has previously been characterized in detail (McClarty et al., 1986a,b, 1987). SC2 cells were routinely cultured at 37 °C on plastic tissue culture plates (Lux Scientific) in α-minimal essential medium (α-MEM) (Flow Laboratories) supplemented with antibiotics and 10% (v/v) fetal calf serum (Gibco). Unless otherwise stated, $SC2^-$ and $SC2^+$ refer to drug-resistant cells that were cultured in the absence and presence of 5 mM hydroxyurea, respectively. The growth medium (with or without hydroxyurea) was routinely replaced with fresh medium every 24 to 48 h during experimentation. $SC2^-$ cells had a doubling time of approximately 25 h, and $SC2^+$ cells doubled every 32 h. $SC2^-$ cells were routinely cultured in the absence of hydroxyurea for at least 2 weeks prior to experimentation.

RNA Dot Blot Analysis. Analysis of relative mRNA levels for M1 and M2 protein was carried out by using the cytoplasmic dot hybridization technique (White & Bancroft, 1982). RNA was denatured by incubation at 60 °C for 15 min in 6 × SSC[1] plus 7.4% formaldehyde. Following dilution with 6 × SSC to the appropriate concentration, RNA was applied to a nitrocellulose membrane using the Bio-Rad dot blot apparatus. Blots were prehybridized and hybridized as previously described (McClarty et al., 1987) using either a *Nco*I-generated fragment containing the cDNA of clone 65 (M1 protein) or the *Pst*I fragment of clone 10 (M2 protein) as hybridization probes (Thelander & Berg, 1986). Blots were washed, and autoradiography was performed as described previously (McClarty et al., 1987).

Immunoblot Assay. Following cell extract preparation, a predetermined quantity of total cell extract protein was heated at 100 °C for 2 min in SDS loading buffer and then analyzed on a 10% linear SDS–polyacrylamide gel as previously described (Engstrom et al., 1979; McClarty et al., 1987). Proteins were then transferred to nitrocellulose membranes by the method of Towbin et al. (1979). This transfer was carried out at 100 V at 4 °C for 1 h. After transfer, membranes were blocked in 50 mM Tris-HCl (pH 7.6) in saline (TBS) containing 0.5% Tween 20 (TBS–Tween) plus 1% bovine serum albumin for 1 h. The membranes were then incubated with either AD 203 anti-M1 mouse monoclonal antibody or JB4 anti-M2 rat monoclonal antibody for 3 h and washed 3 times for 30 min each in TBS–Tween followed by incubation with the appropriate second antibody for 3 h. Goat anti-mouse IgG conjugated with alkaline phosphatase (Sigma) was used for M1 detection, and rabbit anti-rat IgG conjugated with alkaline phosphatase (Sigma) was used for detecting protein M2. Following incubation with second antibody, the blots were washed 3 times for 30 min each in TBS–Tween. Finally, the bound antibodies were detected by the development of the alkaline phosphatase reaction.

Determination of Protein Half-Life and Biosynthesis Rates. The half-life of M1 and M2 proteins in the presence or absence of hydroxyurea was determined by following the decay of prelabeled M1 or M2. Equal numbers of cells were plated for protein half-life determinations, and then cells were cultured for 24 h prior to addition of radiolabeled methionine. Labeling of exponentially growing cells was carried out on plastic tissue culture plates in methionine-free growth medium containing 50 μCi/mL [^{35}S]methionine and 5% (v/v) dialyzed fetal calf serum. Following labeling for 16 h at 37 °C, the "hot" medium was aspirated, and cells were washed 3 times with phosphate-buffered saline (PBS). The chase was carried out at 37 °C with "cold" growth medium containing 10% (v/v) fetal calf serum. Cells were harvested at various time points during the chase by removal from plates with 0.2% buffered trypsin (Difco) solution, centrifuged, washed twice with ice-cold PBS, and then recentrifuged. The resulting cell pellet was resuspended in solubilization buffer [1% Triton X-100, 0.5% sodium deoxycholate, 5 mM EDTA, 250 mM NaCl, 25 mM Tris-HCl, pH 7.5, and 1.0 mM phenylmethanesulfonyl fluoride (PMSF; Boehringer, Manneheim)]. Immunoprecipitation was carried out by using saturating amounts of AD 203 anti-M1 mouse monoclonal antibody (Enstrom et al.,

[1] Abbreviations: SDS, sodium dodecyl sulfate; TBS–Tween, 50 mM Tris-HCl, pH 7.6, in 150 mM NaCl and 0.5% Tween 20; 1 × SSC, 0.15 M NaCl and 15 mM NaCl/citrate, pH 7.0.

1984) or JB4 anti-M2 rat monoclonal antibody (Y. Engstrom, unpublished observations) according to the method of Firestone et al. (1982) except only one round of immunoprecipitation was performed. Formalin-fixed staph A cells (Pansorbin) were purchased from Calbiochem. The resulting precipitated products were analyzed on 10% SDS–polyacrylamide gels. After equilibration in 25 mM Tris-HCl (pH 8.3), 25 mM glycine gels were dried, and the band of protein M1 or M2 was visualized by autoradiography. Dried gels were exposed to Kodak X-OMAT AR film for 12–36 h. Protein M1 or M2 bands were scanned, and peak areas were quantitated (Beckman DU-8 spectrometer).

The apparent rate of protein M1 or M2 biosynthesis in the presence or absence of hydroxyurea and/or iron was determined by measuring the incorporation of [^{35}S]methionine into the two enzyme subunits. Cells (9×10^5) were biosynthetically labeled at 37 °C with a 30-min pulse of 500 μCi of [^{35}S]-methionine/mL of methionine-free medium plus 5% (v/v) dialyzed fetal calf serum. Immediately following the pulse label, cells were harvested and lysed as described above. For measurement of radioactivity incorporated into total soluble protein, 10-μL aliquots of the labeled cell extracts were precipitated with 10% trichloroacetic acid followed by filtration through Whatman GF/A filters and liquid scintillation counting. The remainder of the labeled cell extract was used for determination of radioactivity incorporated into newly synthesized M1 or M2 protein. Immunoprecipitation with saturating amounts of M1 or M2 monoclonal antibody, SDS gel electrophoresis, autoradiography and densitometry were carried out as described above.

Protein Determinations. Concentrations of protein were estimated by using the Bio-Rad protein determination kit (Technical Bulletin 1051) with bovine serum albumin as the standard.

Results

Effect of Hydroxyurea Concentration on M1 and M2 Protein and mRNA Levels. In a previous study, we reported that ribonucleotide reductase activity in the SC2 mutant cell line increased in a drug concentration dependent manner when cells were cultured in the presence of hydroxyurea (McClarty et al., 1986a). In order to determine whether this drug concentration dependent increase in activity was due to changes in the level of protein M1, M2, or both M1 and M2, we cultured SC2 cells previously grown out of drug in the presence of various concentrations of hydroxyurea for 3 days and then examined M1 and M2 protein levels by Western blot analysis. We have previously shown by flow cytometry that there are only slight variations in the cell cycle distribution when SC2 cells are grown in the presence of 5 mM hydroxyurea (McClarty et al., 1987). Furthermore, even when cultured in the presence of 10 mM hydroxyurea, there is only a 6% increase in the number of cells in S phase (data not shown). Therefore, the observed increase in reductase activity is not a result of a significantly larger proportion of the cell population being in S phase. The effect of various concentrations (0, 1, 5, and 10 mM) of hydroxyurea on the level of proteins M1 and M2 in SC2 cells is shown in Figure 1A,B, respectively. The results clearly indicate that there is an increase in the level of both proteins M1 and M2 in response to hydroxyurea and the elevations occur in a drug concentration dependent manner.

In order to determine if the level of M1 and M2 mRNAs changed when SC2 cells were grown in the presence of various concentrations of hydroxyurea, a quantitative cytoplasmic dot blot analysis was carried out. Results of dot hybridization analysis, using cytoplasmic extract prepared from SC2 cells grown in the absence and presence of 1, 5, and 10 mM hydroxyurea, probed with a M1- and M2-specific cDNA (Thelander & Berg, 1986), are shown in Figure 1C. In agreement with our previous findings (McClarty et al., 1987), the results indicated that the levels of both M1 and M2 transcripts were essentially unchanged when the mutant cell line was grown in the presence of hydroxyurea.

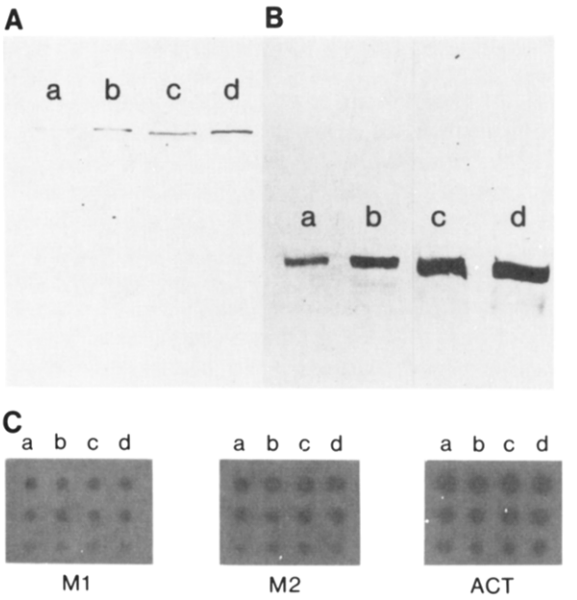

FIGURE 1: Effect of hydroxyurea on cellular M1 and M2 protein and mRNA levels. Cell extract preparation, Western blot procedure, and cytoplasmic dot hybridization were carried out as described under Materials and Methods. (A) Western blot analysis for protein M1; 25 μg of cell extract protein was loaded in each lane. (B) Western blot analysis for protein M2; 10 μg of cell extract protein was loaded in each lane. Individual lanes represent cell extract protein prepared from SC2 cells grown in the (a) absence and presence of (b) 1, (c) 5, and (d) 10 mM hydroxyurea for 3 days. (C) Cytoplasmic dot hybridization for M1, M2, and β-actin mRNAs in SC2 cells grown in the (a) absence and presence of (b) 1, (c) 5, and (d) 10 mM hydroxyurea for 3 days. Cytoplasmic extract was prepared from SC2 cells and treated with 7.4% formaldehyde as previously described (White & Bancroft, 1982). Treated extract was then spotted in serial 2-fold dilutions onto nitrocellulose followed by hybridization with either an M1 cDNA fragment, an M2 cDNA fragment, or a β-actin cDNA probe to quantitate the relative amounts of M1 and M2 mRNAs in each preparation. The amount of cytoplasmic extract present in the least dilute spot corresponds to 2×10^5 cells for lanes a–d for the M1 blot, 5×10^4 cells for lanes a–d for the M2 blot, and 5×10^4 cells for lanes a–d for the actin blot. The autoradiograms were exposed for 24 h for the M1 blot and 12 h for both M2 and β-actin at −70 °C with intensifying screens.

Kinetics of Protein M1 and M2 Induction. To gain additional information about the mechanism responsible for the hydroxyurea-induced elevation of ribonucleotide reductase in the SC2 cell line, we decided to investigate the kinetics of protein M1 and M2 induction. SC2 cells that had been cultured in the absence of hydroxyurea for several days were returned to medium containing 5 mM drug, and at various time points, cells were harvested, extracts were prepared, and M1 and M2 protein levels were analyzed by Western blot. Induction of the M1 subunit occurs quite slowly with no significant increase in protein until cells had been in the presence of hydroxyurea for at least 6 h (Figure 2A, compare lanes a and d). In contrast, protein M2 appears to respond much more rapidly to hydroxyurea treatment with noticeable increases in protein as early as 1–3 h after drug addition (Figure 2B, compare lanes a, b, and c). It is clear from the results that even after being cultured in the presence of hydroxyurea for 24 h the induction of both proteins M1 and M2 is not complete

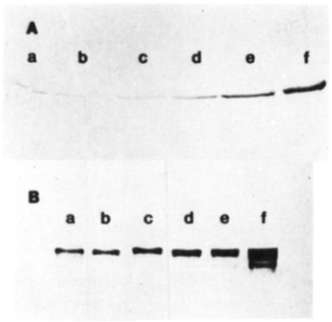

FIGURE 2: Kinetics of protein M1 and M2 induction. Cell extract preparation and Western blot procedure were carried out as described under Materials and Methods. Western blot analysis for (A) protein M1 and (B) protein M2 in SC2 cells cultured in the (a) absence and presence of 5 mM hydroxyurea for (b) 1 h, (c) 3 h, (d) 6 h, (e) 24 h, and (f) continuously (>2 weeks). For protein M1, 25 μg of cell extract protein was loaded in each lane, and 10 μg of cell extract protein was loaded in each lane for protein M2.

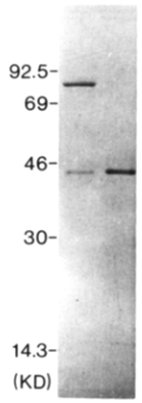

FIGURE 3: M1 and M2 immunoprecipitation. Immunoprecipitation of protein M1 and M2 from metabolically labeled cell extracts was carried out as described under Materials and Methods and in Firestone et al. (1982). The specificity of the M1 immunoprecipitation is shown on the left and the M2 immunoprecipitation on the right. The band at 44 000 daltons in the M1 immunoprecipitation has been shown to be protein M2 that coprecipitates with protein M1 as a holoenzyme.

since SC2 cells grown continuously (>2 weeks) in the presence of drug have accumulated significantly more M1 and M2 (Figure 2A,B, compare lanes e and f).

Effect of Hydroxyurea on the Degradation Rates of Proteins M1 and M2. The increases in protein M1 and M2 levels in SC2 cells grown in the presence of hydroxyurea, in the absence of elevations in their corresponding mRNA levels, suggest that the protein increases occur by a posttranscriptional mechanism. It seemed possible that an alteration in the stability of M1 and M2 protein could account for the hydroxyurea-induced increases seen for both subunits. In order to examine this possibility, the decay rate of [^{35}S]methionine-labeled M1 and M2 protein was compared in SC2 cells grown in the presence and absence of hydroxyurea. SC2$^-$ and SC2$^+$ cells were labeled for 16 h with [^{35}S]methionine, followed by culturing in chase medium free of label; then at various intervals, cells were harvested, and immunoprecipitation was carried out using monoclonal antibodies specific for protein M1 or M2. The specificity of the immunoprecipitation of metabolically labeled M1 (M_r 85 000) and M2 (44 000) is shown in Figure 3. Following sodium dodecyl sulfate (SDS) gel electrophoresis and autoradiography, quantitative determinations were carried out by using a Beckman DU-8 scanning spectrophotometer to measure the intensity of the labeled M1 or M2 band at the various time intervals. The results of this pulse–chase experiment indicated a half-life of 15.25 h for

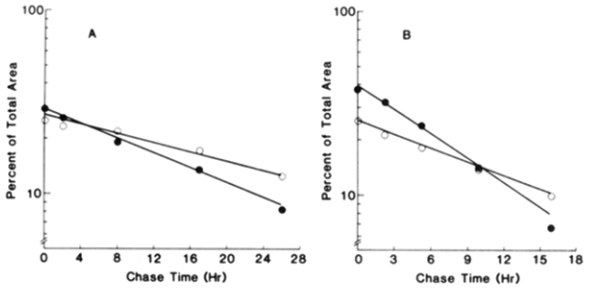

FIGURE 4: Half-life determination for proteins M1 and M2. Pulse–chase analysis of (A) protein M1 and (B) protein M2 half-life; (●) SC2 cells grown in the absence of hydroxyurea and (○) SC2 cells grown in the presence of 5 mM hydroxyurea >2 weeks. The $t_{1/2}$ (half-life) is determined from the time of chase when half the initial labeled M1 or M2 protein is remaining. Following immunoprecipitation with M1 or M2 monoclonal antibodies and SDS gel electrophoresis, the labeled proteins were detected by autoradiography and quantitated by densitometric analysis as described under Materials and Methods. The y-axis scale is logarithmic.

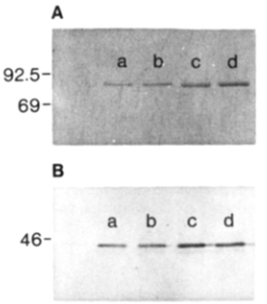

FIGURE 5: Effect of hydroxyurea and iron on the rate of M1 and M2 biosynthesis. SC2 cells were cultured in the absence or presence of 5 mM hydroxyurea and/or 150 μM ferric ammonium citrate prior to pulse-labeling with [^{35}S]methionine for 30 min. Following labeling, cells were washed and lysed, and then the supernatant was immunoprecipitated with anti-M1 or anti-M2 monoclonal antibody and subjected to SDS–polyacrylamide gel electrophoresis and autoradiography as described under Materials and Methods. (A) Immunoprecipitation for protein M1 and (B) immunoprecipitation for protein M2 from (a) SC2 cells + no treatment, (b) SC2 cells + 150 μM ferric ammonium citrate for 3 h, (c) SC2 cells + 5 mM hydroxyurea for >2 weeks, and (d) SC2 cells + 150 μM ferric ammonium citrate for 3 h and 5 mM hydroxyurea for >2 weeks.

protein M1 and of 6.75 h for protein M2 in SC2 cells grown in the absence of hydroxyurea. When SC2 cells were cultured in the presence of hydroxyurea, the half-lives of both proteins M1 and M2 increased by approximately 2-fold to 25.75 and 12.25 h, respectively (Figure 4A,B).

Rates of Protein M1 and M2 Biosynthesis. Even though M1 and M2 mRNA levels are unchanged in SC2 cells cultured in the presence of hydroxyurea, altered rates of M1 and M2 polypeptide biosynthesis may also contribute to the regulation of M1 and M2 protein levels by hydroxyurea. To compare the rates of biosynthesis of M1 and M2 in SC2 cells grown in the presence and absence of hydroxyurea, cells were pulse-labeled with [^{35}S]methionine for 30 min, and then cell lysates were prepared and immunoprecipitated with M1 and M2 monoclonal antibody followed by SDS gel electrophoresis and autoradiography. The results of such an experiment are shown in Figure 5. The rates of biosynthesis of both protein M1 (Figure 5A, compare lanes a and c) and protein M2 (Figure 5B, compare lanes a and c) have increased in the SC2 cell line grown in the presence of hydroxyurea when compared to the same cells grown in the absence of drug. The result shown is representative of four such experiments. The average of the four densitometric quantitations of the labeled M1 and M2 immunoprecipitates under the different experimental conditions is presented in Table I. For easy comparison, the

Table I: Effect of Hydroxyurea and Iron on the Rates of Biosynthesis of Proteins M1 and M2[a]

hydroxyurea treatment[b]	ferric ammonium citrate treatment[c]			
	protein M1		protein M2	
	−	+	−	+
−	1.00	1.11 ± 0.14	1.00	0.97 ± 0.08
+	3.30 ± 0.39	4.26 ± 0.55	1.92 ± 0.15	1.26 ± 0.13

[a] The average (±SE) of four densitometric quantitations of labeled M1 and M2 immunoprecipitates under the different experimental conditions. For easy comparison, the values have been normalized such that the level of [^{35}S]methionine incorporated into protein M1 or M2 in SC2 cells grown in the absence of hydroxyurea and iron has been arbitrarily set at 1. See text and Materials and Methods for further details. [b] 5 mM for >2 weeks. [c] 150 μM for 3 h.

Table II: Kinetics of the Hydroxyurea Effect on the Rates of Biosynthesis of Proteins M1 and M2[a]

time after hydroxyurea addition (h)	protein M1	protein M2
	1.00	1.00
1	0.99 ± 0.04	0.94 ± 0.02
3	1.02 ± 0.04	1.04 ± 0.01
6	1.06 ± 0.02	0.99 ± 0.06
24	1.90 ± 0.06	1.27 ± 0.05
48	2.43 ± 0.10	1.73 ± 0.03
>2 weeks	3.02 ± 0.08	1.93 ± 0.04

[a] The average (±SE) of two densitometric quantitations of labeled M1 and M2 immunoprecipitates at various time points following addition of hydroxyurea to the growth medium. For easy comparison, the values have been normalized such that the level of [^{35}S]methionine incorporated into protein M1 or M2 in SC2 cells grown in the absence of hydroxyurea has been arbitrarily set at 1. See text and Materials and Methods for further details.

values have been normalized such that the level of [^{35}S]-methionine incorporated into protein M1 or M2 in SC2 cells grown in the absence of hydroxyurea has been arbitrarily set at 1. It is important to note that little or no effect on total protein synthesis was observed after treatment with hydroxyurea. SC2 cells incorporated 6.4×10^4 cpm/μg of protein whereas SC2$^+$ cells incorporated 6.1×10^4 cpm/μg of protein during the 30-min [^{35}S]methionine labeling period.

In order to determine how rapidly the increase in the rate of M1 and M2 biosynthesis occurs, in response to hydroxyurea, a kinetic study was conducted. SC2$^-$ cells were cultured in the presence of hydroxyurea for various lengths of time prior to pulse-labeling with [^{35}S]methionine for 30 min. Immediately following the pulse-label, cells were harvested, and then immunoprecipitation, SDS gel electrophoresis, and autoradiography were performed. Densitometric quantitation of the metabolically labeled M1 and M2 immunoprecipitates at the various time points was then carried out. The average peak area of two such determinations for each time point is shown in Table II. Once again, the values have been normalized such that the level of [^{35}S]methionine incorporated into protein M1 or M2 in SC2 cells grown in the absence of hydroxyurea has been set at 1. The rate of biosynthesis of both proteins M1 and M2 responds slowly to the presence of hydroxyurea with neither subunit showing any significant increase in synthesis rate until 24 h after drug addition. Thereafter, the rates of M1 and M2 biosynthesis steadily increased until they reached the level occurring in SC2 cells growing continuously (>2 weeks) in the presence of hydroxyurea.

Effect of Iron on Protein M1 and M2 Levels and Rates of Biosynthesis. Ferritin is the major iron storage protein in eukaryotic cells (Theil, 1987). Biosynthetic rates of ferritin and cellular ferritin levels are regulated by changes in iron availability, and this regulation occurs mainly through post-

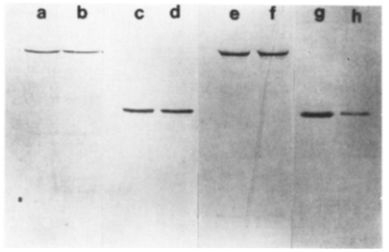

FIGURE 6: Effect of hydroxyurea and iron on total cellular M1 and M2 protein levels. Cell extract preparation and Western blot procedure were carried out as described under Materials and Methods. SC2 cells were grown in the absence or presence of 5 mM hydroxyurea and/or 30 μM ferric ammonium citrate for the indicated period of time prior to cell extract preparation. Western blot analysis for protein M1 a, b, e, and f and protein M2 c, d, g, and h. (a) SC2 cell extract, no treatment, 40 μg of protein; (b) SC2 cell extract, 30 μM ferric ammonium citrate for 3 days, 40 μg of protein; (c) SC2 cell extract, no treatment, 15 μg of protein; (d) SC2 cell extract, 30 μM ferric ammonium citrate for 3 days, 15 μg of protein; (e) SC2 cell extract, 5 mM hydroxyurea >2 weeks, 25 μg of protein; (f) SC2 cell extract, 5 mM hydroxyurea >2 weeks + 30 μM ferric ammonium citrate for 3 days, 25 μg of protein; (g) SC2 cell extract, 5 mM hydroxyurea >2 weeks, 10 μg of protein; (h) SC2 cell extract, 5 mM hydroxyurea >2 weeks + 30 μM ferric ammonium citrate for 3 days, 10 μg of protein.

transcriptional mechanisms (Aziz & Munro, 1986; Mattia et al., 1986; Theil, 1987). Protein M2 contains stoichiometric amounts of non-heme iron which is essential for enzyme activity (Thelander et al., 1985). It has been shown that hydroxyurea inhibits ribonucleotide reductase by destroying the tyrosine free radical of protein M2 (Graslund et al., 1982); however, it is also known that hydroxyurea has iron chelating properties (Young et al., 1967). It seemed possible that the effect hydroxyurea has on ribonucleotide reductase levels and biosynthesis rates could be a result of changes in iron availability when cells are cultured in the presence of the drug. In order to test this hypothesis, we decided to determine what effect iron has on M1 and M2 protein levels and rates of biosynthesis in SC2 cells grown in the presence or absence of hydroxyurea.

SC2$^-$ and SC2$^+$ cells were cultured in the presence of various concentrations of ferric ammonium citrate for 3 days; then total cellular levels of proteins M1 and M2 were determined by Western blot analysis. We found that 30 μM ferric ammonium citrate was optimal, and as can be seen in Figure 6, at this concentration, iron had no obvious effect on M1 levels in SC2 cells whether they were cultured in the presence or absence of hydroxyurea (Figure 6, compare lanes a and b as well as lanes e and f). Similarly, iron had little or no effect on protein M2 levels in SC2 cells cultured in the absence of hydroxyurea (Figure 6, compare lanes c and d); however, the presence of iron caused a dramatic decrease in protein M2 levels in SC2 cells cultured in the presence of hydroxyurea (Figure 6, compare lanes g and h).

The effect of iron on the rate of protein M1 and M2 biosynthesis in SC2$^-$ and SC2$^+$ cells is shown in Figure 5. For these experiments, SC2$^-$ and SC2$^+$ cells were cultured in the presence of 150 μM ferric ammonium citrate for 3 h, and then the cells were pulse-labeled for 30 min with [^{35}S]methionine. Immediately following labeling the cells were harvested and cellular extracts were immunoprecipitated with M1 or M2 monoclonal antibody followed by SDS gel electrophoresis and autoradiography. Iron had no significant effect on the rate of synthesis of either protein M1 or protein M2 in SC2 cells grown in the absence of hydroxyurea (Figure 5A, compare lanes a and b; and Figure 5B, compare lanes a and b). In contrast, when iron was added to SC2 cells growing in the

presence of hydroxyurea, the rate of biosynthesis of both M1 and M2 was affected. Interestingly, the rate of M1 synthesis appeared to increase slightly whereas the rate of M2 biosynthesis showed a small but consistent decrease (Figure 5A, compare lanes c and d, as well as Figure 5B, compare lanes c and d). The average of densitometric quantitations from four such experiments is presented in Table I.

Discussion

In the past, we have shown that the SC2 mutant cell line used in this study induces ribonucleotide reductase activity when grown in the presence of the chemotherapeutic agent hydroxyurea (McClarty et al., 1986a, 1987). To better understand the mechanisms that underlie the altered activity in the mutant cell line and, perhaps, to learn something thereby about the control of ribonucleotide reductase activity in normal cells, we examined several factors, the change of which could plausibly explain the drug-induced increase in ribonucleotide reductase activity. The increase in reductase activity in the SC2 cell line, upon exposure to hydroxyurea, has been shown to be both time and drug concentration dependent (McClarty et al., 1986a). In order to determine whether these time- and dose-dependent increases were a result of elevations in protein M1, M2, or both M1 and M2, we estimated the cellular levels of both subunits by Western blot analysis. Our results clearly demonstrated that both the M1 and M2 subunits of ribonucleotide reductase respond to hydroxyurea in a drug concentration dependent manner (Figure 1A,B). Both enzyme components are substantially elevated in drug concentrations as low as 1 mM, and both subunits continue to increase even in concentrations of hydroxyurea as high as 10 mM. This result is in complete agreement with our earlier observation that enzyme activity continued to rise in response to increasing drug concentrations (McClarty et al., 1986a). It is also evident from our results that the elevations in M1 and M2 proteins occur in the absence of a corresponding change in mRNA levels for either subunit (Figure 1C).

The results of kinetic studies indicated that protein M2 levels rise much more rapidly, within 1–3 h, than protein M1 which showed no substantial increase until sometime between 6 and 24 h, after hydroxyurea addition (Figure 2A,B). It is interesting to note that we had previously shown that no significant elevation in ribonucleotide reductase activity takes place until sometime between 6 and 18 h after drug addition (McClarty et al., 1986a). Taken together, the data strongly suggest that, in contrast to wild-type cells where protein M2 is normally limiting (Eriksson et al., 1984), the M1 subunit is limiting in this mutant cell line and therefore regulates the level of ribonucleotide reductase activity. Although we cannot exclude the possibility that posttranslational modification of either the M1 or the M2 subunit may also play a role in the regulation of ribonucleotide reductase activity by hydroxyurea, our data support the hypothesis that drug-induced changes in enzyme activity occur mainly as a result of alterations in the amount of enzyme protein.

The results presented in this and in an earlier report (McClarty et al., 1987) clearly indicate that hydroxyurea can modulate ribonucleotide reductase expression posttranscriptionally. In this study, we directly determined the rates of both synthesis and degradation of proteins M1 and M2 by measuring the radioactivity of [^{35}S]methionine-labeled M1 and M2 isolated from crude extracts by immunoprecipitation and SDS–polyacrylamide gel electrophoresis. The isolation procedure was satisfactory (Figure 3), and therefore, this method gave accurate results. We have observed that the half-lives of proteins M1 and M2 in the SC2 mutant cell line grown out of drug are unchanged from those determined for both subunits in the parental wild-type cell line. Measurements of the rate of turnover of proteins M1 and M2 in the mutant cell line grown in the presence of hydroxyurea showed that at least part of the drug-induced elevation in both components could be accounted for by an approximate 2-fold increase in half-life (Figure 4A,B). The fact that there was no significant increase in cellular protein M1 levels until 6–24 h after hydroxyurea treatment as compared to 1–3 h for protein M2 (Figure 2A,B) is likely a reflection of the longer half-life of the former. Numerous studies have reported on the effects of various metabolic inhibitors on the rate of degradation of dihydrofolate reductase (Alt et al., 1976; Domin et al., 1982; Cowan et al., 1986), ornithine decarboxylate (Persson et al., 1985; Pegg, 1986), and *S*-adenosylmethionine decarboxylase (Pegg, 1984, 1986; Shirahata & Pegg, 1985; Persson et al., 1985). The mechanisms responsible for these stabilizations are not yet fully understood; however, it has been suggested that the presence of an inhibitor in the active site renders the enzyme less susceptible to proteolytic degradation (Schimke, 1973). Such a mechanism cannot explain the hydroxyurea-induced stabilization of ribonucleotide reductase since it is not a substrate analogue and the site of action of the drug is known to be the M2 component tyrosyl free radical (Akerblom et al., 1981).

Changes in protein M1 and M2 stability account only in part for the regulation of ribonucleotide reductase levels by hydroxyurea. By measuring the rate of M1 and M2 protein biosynthesis directly in pulse-labeling experiments, we have shown that an effect at the translational level, possibly increased translational efficiency, assumes a significant role in the hydroxyurea-induced elevations of proteins M1 and M2 (Figure 5A,B, Table I). In this connection, it is important to note that total cellular protein synthesis rates, measured by [^{35}S]methionine incorporation into proteins, were essentially unaffected by hydroxyurea treatment which attests to the selectivity of increased M1 and M2 biosynthesis rates in the presence of hydroxyurea. Inhibitors of ornithine decarboxylase activity alter polyamine pools, and accumulating evidence from several laboratories indicates that it is the polyamines that regulate ornithine decarboxylase biosynthesis (Kahana & Nathans, 1985; Kanamoto et al., 1986; Dircks et al., 1986; Holtta & Pohjankelto, 1986; McConlogue et al., 1986). Recently, it has been shown that polyamines have a direct inhibitory effect on the in vitro translation of mRNA for ornithine decarboxylase (Kameji & Pegg, 1987). Hydroxyurea is known to cause alterations in deoxyribonucleotide pools (Skoog & Nordenskjold, 1971; Nicander & Reichard, 1985). Whether or not any of the four deoxyribonucleotides can directly, or indirectly, through an unknown intermediate, affect M1 or M2 protein biosynthesis has not yet been elucidated.

Results of kinetic studies indicate that the increase in the rate of M1 and M2 biosynthesis in response to hydroxyurea occurs slowly with no significant effect until 24 h after the initial drug treatment (Table II). The substantial increase in cellular protein M1 and M2 levels seen in SC2 cells continually (>2 weeks) grown in the presence of hydroxyurea compared to cells exposed to drug for just 24 h (Figure 2A,B, compare lanes e and f) is probably a result of this slow responding increase in the biosynthesis rate of both proteins.

The effect that exogenously added iron has on M1 and M2 biosynthesis was experimentally explored in an attempt to gain some insight into the mechanism behind the translational regulation of ribonucleotide reductase. It has been known for quite a while that iron regulates the synthesis of ferritin, in many cells, at the level of translation (Zahringer et al., 1976;

Aziz & Munro, 1986; Mattia et al., 1986; Theil, 1987). This regulation is believed to be mediated by a protein, which presumably binds iron and then interacts with a specific 5′ leader sequence of ferritin mRNA (Aziz & Munro, 1987; Hentze et al., 1987). The results presented in this study indicate that exogenously added iron has no significant effect on protein M1 levels; however, iron treatment does lead to a decrease in cellular protein M2 levels but only when SC2 cells are grown in the presence of hydroxyurea (Figure 6). It is interesting to note that iron treatment leads to an uncoupling of the M1 and M2 response to hydroxyurea; i.e., M1 protein levels as estimated by Western blot were unaffected by iron whereas M2 levels decreased. This is the first preliminary indication that M1 and M2 may respond to independent regulatory signals. In addition, it should be mentioned that iron treatment of cells, growing either in the absence or in the presence of hydroxyurea, had no significant effect on M1 or M2 mRNA levels (data not shown). However, iron did have a small but reproducible effect on the biosynthesis rate of both proteins M1 and M2, but once again, this effect was only seen when SC2 cells were grown in the presence of hydroxyurea (Figure 5 and Table I). The rate of M2 biosynthesis was decreased by iron treatment, not an unexpected result given the lower cellular protein M2 levels seen under similar conditions. Surprisingly, protein M1 biosynthesis rates were slightly elevated in the presence of iron, a result which is not reflected in cellular protein M1 levels which were unaffected by iron treatment. We have not yet determined if the half-lives of proteins M1 and M2 are altered by iron treatment. Given that the effects of iron are only seen when cells are cultured in the presence of hydroxyurea suggests that the metal may exert its action through hydroxyurea rather than directly itself. Clearly, more work is required before it will be possible to determine the exact mechanism responsible for these effects. Interestingly, Fontecave et al. (1987) have recently described a ferric iron reductase in *Escherichia coli* that participates in the generation of the free radical of ribonucleotide reductase. They suggested that it may form part of a biological mechanism that regulates the activity of ribonucleotide reductase by establishing the content of tyrosyl radical in the enzyme.

In conclusion, we have shown that hydroxyurea increases the levels of ribonucleotide reductase without increasing the mRNA levels of either the M1 or the M2 subunit. Our results indicate that protein M1 and M2 levels rise proportionately with increasing hydroxyurea concentration. These elevations are brought about not only by increasing the stabilization of both proteins against degradation but also by increasing their rates of biosynthesis. Furthermore, these changes are consistent with elevations in enzyme activity observed with cells grown in the presence of hydroxyurea. This is also the first observation that iron treatment affects the levels of M1 and M2 protein differently in the presence of hydroxyurea, indicating that the two enzyme components may respond to different regulatory signals.

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We note with regret the untimely death of Dr. Bill Lewis and acknowledge his past contributions to the field of drug resistance.

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REFERENCES

Akerblom, L., Ehrenberg, A., Graslund, A., Lankinen, H., Reichard, P., & Thelander, L. (1981) *Proc. Natl. Acad. Sci. U.S.A. 78*, 2159–2163.

Alt, F., Kellems, R., & Schimke, R. (1976) *J. Biol. Chem. 251*, 3063–3074.

Aziz, N., & Munro, H. (1986) *Nucleic Acids Res. 14*, 915–927.

Aziz, N., & Munro, H. (1987) *Proc. Natl. Acad. Sci. U.S.A. 84*, 8478–8482.

Cocking, J., Tonin, P., Stockoe, N., Wensing, E., Lewis, W., & Srinivason, P. (1987) *Somat. Cell Mol. Genet. 13*, 221–233.

Cowan, K., Goldsmith, M., Ricciardone, M., Levine, R., Rubalcaba, E., & Jolivet, J. (1986) *Mol. Pharmacol. 30*, 69–76.

Dircks, L., Grens, A., Slezynger, T., & Scheffler, I. (1986) *J. Cell. Physiol. 126*, 371–378.

Domin, B., Grill, S., Bastow, K., & Cheng, Y.-C. (1982) *Mol. Pharmacol. 21*, 478–482.

Engstrom, Y., Ericksson, S., Thelander, L., & Akerman, M. (1979) *Biochemistry 18*, 2941–2948.

Engstrom, Y., Rozell, B., Hansson, H., Stenne, S., & Thelander, L. (1984) *EMBO J. 3*, 863–867.

Engstrom, Y., Eriksson, S., Jildevik, I., Skog, S., Thelander, L., & Tribukait, B. (1985) *J. Biol. Chem. 260*, 9114–9116.

Eriksson, S., Graslund, A., Skog, A., Thelander, L., & Trikbukait, B. (1984) *J. Biol. Chem. 259*, 11695–11700.

Firestone, G., Payvan, F., & Yamamoto, K. (1982) *Nature (London) 300*, 221–225.

Fontecave, M., Eliasson, R., & Reichard, P. (1987) *J. Biol. Chem. 262*, 12325–12331.

Graslund, A., Ehrenberg, A., & Thelander, L. (1982) *J. Biol. Chem. 257*, 5711–5715.

Holtta, E., & Pohjanpelto, P. (1986) *J. Biol. Chem. 261*, 9502–9508.

Hentze, M., Wright-Caughman, S., Rouault, T., Barriochanol, J., Dancis, A., Harford, J., & Klausner, R. (1987) *Science (Washington, D.C.) 238*, 1570–1573.

Kahana, C., & Nathans, D. (1985) *J. Biol. Chem. 260*, 15390–15393.

Kameji, T., & Pegg, A. E. (1987) *J. Biol. Chem. 262*, 2427–2430.

Kanamoto, R., Utsunomiya, K., Kameji, T., & Hayashi, S.-I. (1986) *Eur. J. Biochem. 154*, 539–544.

Mattia, E., Josie, D., Ashwell, G., Klausner, R., & Van Renswoude, J. (1986) *J. Biol. Chem. 261*, 4587–4593.

McClarty, G., Chan, A., & Wright, J. A. (1986a) *Somat. Cell Mol. Genet. 12*, 121–131.

McClarty, G., Chan, A., & Wright, J. A. (1986b) *Cancer Res. 46*, 4516–4521.

McClarty, G., Chan, A., Engstrom, Y., Wright, J. A., & Thelander, L. (1987) *Biochemistry 26*, 8004–8011.

McConlogue, L., & Coffino, P. (1983) *J. Biol. Chem. 258*, 8384–8388.

McConlogue, L., Dana, S., & Coffino, P. (1986) *Mol. Cell. Biol. 6*, 2865–2871.

Nicander, B., & Reichard, P. (1985) *J. Biol. Chem. 260*, 5376–5381.

Pegg, A. E. (1984) *Biochem. J. 224*, 29–38.

Pegg, A. E. (1986) *Biochem. J. 234*, 249–262.

Persson, L., Oredsson, M., Anehus, S., & Heby, O. (1985) *Biochem. Biophys. Res. Commun. 131*, 239–245.

Schimke, R. T. (1973) *Adv. Enzymol. Relat. Areas Mol. Biol. 37*, 135–187.

Shirahata, A., & Pegg, A. E. (1985) *J. Biol. Chem. 260*, 9583–9588.

Skoog, L., & Nordenskjold, B. (1971) *Eur. J. Biochem. 19*, 81–89.

Tagger, A. Y., Boux, J., & Wright, J. A. (1987) *Biochem. Cell Biol. 65*, 925–929.
Theil, E. C. (1987) *Annu. Rev. Biochem. 56*, 289–315.
Thelander, L., & Reichard, P. (1979) *Annu. Rev. Biochem. 48*, 133–158.
Thelander, L., & Berg, P. (1986) *Mol. Cell. Biol. 6*, 3433–3442.
Thelander, L., Eriksson, S., & Akerman, M. (1980) *J. Biol. Chem. 255*, 7426–7432.
Thelander, M., Graslund, A., & Thelander, L. (1985) *J. Biol. Chem. 260*, 2737–2741.
Towbin, H., Staehlin, T., & Gordon, J. (1979) *Proc. Natl. Acad. Sci. U.S.A. 76*, 4350–4354.
White, B., & Bancroft, C. (1982) *J. Biol. Chem. 257*, 8569–8572.
Wright, J. A. (1988) *Int. Encycl. Pharmacol. Ther.* (in press).
Wright, J. A., Alam, T., McClarty, G., Tagger, A. Y., & Thelander, L. (1987) *Somat. Cell Mol. Genet. 13*, 155–165.
Wright, J. A., McClarty, G. A., Lewis, W. H., & Sirinivasan, P. R. (1988) in *Drug Resistance in Mammalian Cells* (Gupta, R. S., Ed.) CRC, Boca Raton, FL (in press).
Young, C., Schochetman, G., Hodas, S., & Balis, E. (1967) *Cancer Res. 27*, 535–540.
Zahringer, J., Baliga, B., & Munro, H. (1976) *Proc. Natl. Acad. Sci. U.S.A. 73*, 857–861.

Characterization of the ATP Synthase of *Propionigenium modestum* as a Primary Sodium Pump†

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ABSTRACT: The ATP synthase (F_1F_0) of *Propionigenium modestum* has been purified to a specific ATPase activity of 5.5 units/mg of protein, which is about 6 times higher than that of the bacterial membranes. Analysis by SDS gel electrophoresis indicated that in addition to the five subunits of the F_1 ATPase, subunits of M_r 26 000 (a), 23 000 (b), and 7500 (c) have been purified. The ATPase activity of F_1F_0 was specifically activated about 10-fold by Na^+ ions. The enzyme was strongly inhibited by dicyclohexylcarbodiimide, venturicidin, tributyltin chloride, and azide. After incubation with [^{14}C]dicyclohexylcarbodiimide, about 3–4 mol of the inhibitor was bound per 500 000 g of the enzyme. The radioactive label was specifically bound to subunit c. These subunits form stable aggregates which resist dissociation by SDS at 100 °C. The monomer is formed upon heating with SDS to 121 °C or by extraction of the membranes with chloroform/methanol. The ATP synthase was incorporated into liposomes by a freeze–thaw–sonication procedure. The reconstituted proteoliposomes catalyzed the transport of Na^+ ions upon ATP hydrolysis. The transport was completely abolished by dicyclohexylcarbodiimide. Whereas monensin prevented the accumulation of Na^+ ions, the uptake rate was stimulated 4–5-fold in the presence of valinomycin or carbonyl cyanide *m*-chlorophenylhydrazone. These results indicate an electrogenic Na^+ transport and also that it is a primary event and not accomplished by a H^+-translocating ATP synthase in combination with a Na^+/H^+ antiporter.

The strictly anaerobic bacterium *Propionigenium modestum* grows from the fermentation of succinate to propionate and CO_2 (Schink & Pfennig, 1982). The pathway of succinate fermentation involves the intermediates succinyl-CoA, (*R*)- and (*S*)-methylmalonyl-CoA, and propionyl-CoA. The mechanism of ATP synthesis in this organism deserves special attention (Hilpert et al., 1984): the small free energy change of the decarboxylation of succinate to propionate ($\Delta G^{\circ\prime}$ = −20.6 kJ/mol) does not allow a substrate-linked phosphorylation mechanism, and no redox reactions occur which could drive electron-transport phosphorylation. The only step of the fermentation pathway that is sufficiently exergonic for energy conservation is the decarboxylation of (*S*)-methylmalonyl-CoA to propionyl-CoA. The free energy of the decarboxylation reaction is conserved by conversion into an electrochemical Na^+ gradient, which, therefore, provides the only energy source for ATP synthesis.

Preliminary experiments with bacterial membrane vesicles indicated that the synthesis of ATP was coupled directly to the Na^+ gradient (Hilpert et al., 1984): the vesicles contained an ATPase that was specifically activated by Na^+ ions and catalyzed Na^+ uptake upon ATP hydrolysis that was not sensitive to the uncoupler carbonyl cyanide *p*-(trifluoromethoxy)phenylhydrazone. In order to firmly establish the ATP synthesis mechanism in *P. modestum*, however, the energy-converting enzymes have to be purified and their functions have to be demonstrated with a reconstituted proteoliposomal system.

We have recently shown (Laubinger & Dimroth, 1987) that the Na^+-stimulated ATPase of *P. modestum* is of the F_1F_0 type. The F_1 portion of the enzyme has a very similar subunit pattern as, e.g., the F_1 ATPase of *Escherichia coli*. Upon dissociation of the F_1 moiety from the membrane-bound subunits (F_0), the specific activation of ATP hydrolysis by Na^+ ions was lost; this activation was restored by reconstitution of the enzyme complex from the membrane-bound F_0 part and purified F_1 ATPase. In this paper, we describe the purification

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