ISOLATION AND PROPERTIES OF OSCP AND AN F_1-ATPase BINDING PROTEIN FROM RAT LIVER MITOCHONDRIA - EVIDENCE AGAINST OSCP AS THE LINKING "STALK" BETWEEN F_1 AND THE MEMBRANE

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Summary Oligomycin Sensitivity Conferral Protein (OSCP) and an F_1-ATPase Binding Protein were isolated from F_1-depleted rat liver mitochondrial membrane. Their molecular weights on polyacrylamide gel electrophoresis in the presence of sodium dodecyl sulfate and urea were 22,500 and 8,500 respectively. When incubated with liver TUA (trypsin, urea and ammonia-treated) submitochondrial particles, the binding protein was effective in the binding of F_1 to the particles with the resultant particle-bound ATPase activity not oligomycin sensitive. When OSCP was then incubated with the reconstituted membrane-bound ATPase, its activity became oligomycin sensitive. These results suggest that, first; the binding protein, but not OSCP, connects F_1-ATPase to the membrane of rat liver mitochondria and maybe to the "stalk", if indeed there is a stalk in mitochondrial membrane ATPase complex; and second; the function of OSCP is solely to render the ATPase activity sensitive to oligomycin and other similar inhibitors.

Introduction

F_1-ATPase and OSCP** have been firmly established as protein coupling factors of oxidative phosphorylation in mitochondria. They are required in order to observe ATP-^{32}Pi exchange activity (1,2), energy-linked reduction of NAD^+ by succinate (3, 4) and oxidative phosphorylation (4) when suitable depleted submitochondrial particles are used for reconstitution experiments. In electron micrographs of negatively stained preparations of SMP, bead-like protrusions (referred to as elementary particles) line the inner mitochondrial membrane (5); SMP resolved with respect to F_1-ATPase are devoid of elementary particles, providing evidence that the elementary particles correspond to F_1-ATPase. There

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**Abbreviations: OSCP, oligomycin sensitivity conferral protein; SMP, submitochondrial particles; DTT, dithiothreitol; EDTA, ethylene diamine tetraacetic acid: TUA particles, submitochondrial particles treated with trypsin, urea and sonicated in the presence of ammonia; TCA, trichloroacetic acid; SDS sodium dodecyl sulfate; BSA, bovine serum albumin; DCCD, N,N'-dicyclohexylcarbodiimide; BME, β-mercaptoethanol.

is an apparent stalk, visible in some electron micrographs of mitochondria, which connects the elementary particles to the membrane. Since OSCP was extracted from F_1-ATPase-depleted mitochondrial membrane, and it was needed in addition to F_1-ATPase for successful reconstitution of oxidative phosphorylation, it is easy to conclude that OSCP may be the stalk. Indeed, several recent review articles (6, 7) and a widely adopted biochemistry textbook (8) included schematic drawings of the mitochondrial ATPase complex, indicating the stalk to be OSCP. However, when original papers are examined, there is no direct evidence identifying OSCP as the stalk connecting F_1-ATPase to the membrane. In this communication we wish to report the isolation of OSCP and an additional binding protein from rat liver mitochondria; to describe results of experiments which indicate that the binding protein, but not OSCP, is responsible for connecting the F_1-ATPase to the membrane; and to provide evidence that OSCP renders the membrane bound ATPase activity sensitive to oligomycin, or DCCD.

Materials and Methods

Liver mitochondria were isolated from male Sprague-Dawley rats according to the method of Johnson and Lardy (9). Rat liver mitochondrial F_1-ATPase was prepared by the method of Lambeth and Lardy (10).

Crude extract of OSCP containing the binding protein was obtained by the procedure used for OSCP extraction from bovine heart mitochondria (11) with the following modification: 6 g of rat liver mitochondria were suspended in 0.15 M sucrose, 10 mM Tris-SO_4, 3 mM $MgCl_2$, pH 7.8, to a final concentration of 30 mg/ml; 40 ml batches were sonicated in a Raytheon 10.4 Kc sonicator for 4.5 min at maximum power. After removing the unbroken mitochondria by centrifuging at 25,000 x g for 10 min, the SMP were spun down at 100,000 x g for 30 min; after washed with 0.25 M sucrose they were suspended in 0.25 M sucrose, 10 mM Tris-SO_4, 0.2 mM DTT pH 7.5, to a final concentration of 25 mg/ml. The SMP suspension was treated with 3.5 M NaBr (11) twice to remove F_1-ATPase. The depleted particles were washed with 0.25 M sucrose and resuspended in 0.3 M KCl, 1.5 mM EDTA, pH 7.4 to a concentration of 15 mg/ml. To the resulting suspension, one-half volume of freshly prepared 1N NH_4OH was quickly added with efficient stirring (pH was between 11.5 and 11.7). The solution was incubated at 0° for 10 min and centrifuged at 100,000 x g for 15 min. The pH of the supernatant was immediately titrated to 8.0 with 10 N acetic acid. The extraction was repeated once more and the two pH 8.0-titrated supernatants were combined and centrifuged at 100,000 x g for 10 min. The small pellet was discarded. To the supernatant solid ammonium sulfate was added slowly with constant stirring to give a final concentration of 42% saturation. After incubation for at least 60 min the precipitate was spun down at 20,000 x g for 20 min and dissolved in approximately 6 ml of 20 mM Tris-SO_4, pH 8.0.

Purification of OSCP and Binding Protein by CM-sephadex: Crude OSCP was diluted to 150 ml by adding 1 M KCl to make a final concentration of 20 mM KCl, 2 mM Tris-SO_4 and 1 mM EDTA. The solution was added to a 27 ml bed volume of CM-Sephadex C-25 (previously equilibrated with 2 mM KCl, 2 mM Tris-SO_4, pH 8.0).

After stirring the slurry for 60 min and letting it stand for 60 min, the ion-exchanger slurry was poured into a 2 cm diameter column. The column was first washed with 25 ml of 10 mM Tris-SO_4, 0.1 M KCl, 0.1 mM EDTA and 0.1 mM DTT, and then developed with a 110 ml linear gradient of 0.1 M to 0.4 M KCl in the presence of 15 mM Tris-SO_4, 0.1 mM EDTA and 0.1 mM DTT, pH 8.0. The elution rate was approximately 20 ml/hr, and fractions of 2 ml were collected. Active fractions with respect to OSCP activity and F_1-ATPase binding activity were pooled respectively (see Table II) and concentrated either by 60% ammonium sulfate precipitation and dissolved in 20 mM Tris-SO_4, pH 8.0, or by an Amicon UM-05 membrane concentrator and dialysed against 20 mM Tris-SO_4, pH 8.0.

TUA particles from rat liver mitochondria: TUA particles from rat liver mitochondria were prepared by following the procedure used for bovine heart TUA submitochondrial particles (12) except that 30 µg trypsin (Type III bovine pancreas trypsin from Sigma) was used per 10 mg rat liver SMP. The digestion incubation was for 45 min at 30° and 150 µg of trypsin inhibitor/10 mg SMP protein (Type I-S soybean inhibitor from Sigma) was added to stop the reaction. The mixture was immediately cooled to 0°C and an equal volume of 4 M urea was slowly added and the mixture incubated for 45 min at 0°. The treated particles were then spun down at 105,000 x g for 60 min, and washed with 0.25 M sucrose. The pellet was homogenized in 0.25 M sucrose, 2 mM EDTA, pH 7.5 to a final concentration of 20 mg/ml. The suspension was adjusted to pH 10.0 with freshly diluted 1M NH_4OH. 30 ml batches were then exposed to sonic oscillation (Raytheon 10.4 Kc oscillator) for 4 min at maximum power. The resulting suspension was immediately centrifuged at 100,000 x g for 60 min and the pellet was washed with 0.25 M sucrose and finally homogenized in 0.25 M sucrose to yield a suspension of TUA particles, 10-20 mg per ml.

Assays on CM-Sephadex column fractions: Conferral of oligomycin sensitivity on membrane bound F_1 by OSCP was assayed by suspending 200 µg of TUA particles, 5 µg of F_1 and 50 µl of column fractions in 0.1 ml of 0.1 M Tris-SO_4, pH 7.4 at room temperature for 10 min. The mixture was then microcentrifuged for 5 min and the pellet was resuspended in 50 µl of 0.1 M Tris-SO_4, pH 7.4 and added to the assay mixture which included 0.15 ml of 0.1 M Tris-SO_4, 20 µg of pyruvate kinase (Type II from Sigma), 0.675 ml of H_2O and 5 µl of either 95% ethanol (control tube) or oligomycin (66 µg/ml in ethanol). The mixture was incubated 5 min at 30°. 0.1 ml of the substrate mixture (including 0.22 M Tris-SO_4, pH 7.4, 18 mM ATP, 9 mM $MgSO_4$ and 22 mM phosphoenol pyruvate) was added and the mixture was incubated at 30°C for 10 min (13). The reaction was stopped by the addition of 1 ml of 5% TCA. The resulting precipitate was spun down for 5 min in a clinical centrifuge, and 1 ml of the supernatant was assayed for inorganic phosphate by the method of Lohmann and Jendrassik (14). Percentage of inhibition of oligomycin was calculated by comparing the experimental tube with the control tube. Sodium dodecyl sulfate gel electrophoresis was performed by the method of Weber and Osborn (15); samples were preincubated with and without 1% BME as indicated. SDS-Urea gel electrophoresis was done according to the method of Swank and Munkres (16). BSA, bovine ovalbumin, horse heart cytochrome c and trypsin inhibitor were used as standards. Isoelectric focusing was carried out according to the procedure of Vesterberg (17), using 5.5% acrylamide gels in the presence of 8 M urea and pH 3-10 ampholine. The pI was determined by a direct microelectrode measurement. Membrane and soluble proteins were measured by the biuret method (18) and the Lowry method (19) respectively. Amino acid analyses were performed on a Beckman model 120C analyzer by the method of Spackman et al. (20).

Results and Discussion

The results in Figure 1 demonstrate that the ion-exchange column used for purification of bovine heart OSCP (2) was successful in isolating OSCP from rat liver mitochondria. Furthermore, as shown in Figure 1, we have consistently seen

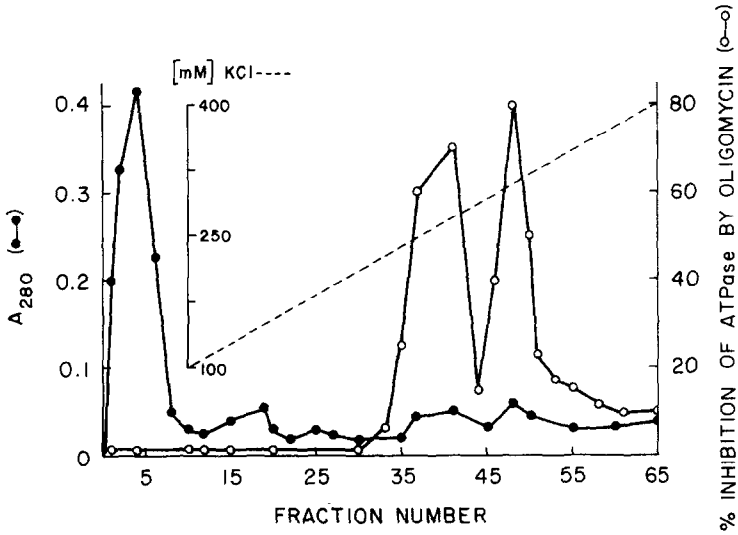

Figure 1 Elution profile from CM-sephadex G25 colunm. Fractions were assayed for OSCP activity (see text for assaying procedure). Fractions 15 to 25 showed stimulating effect of binding added F_1-ATPase to TUA particles (see Table II).

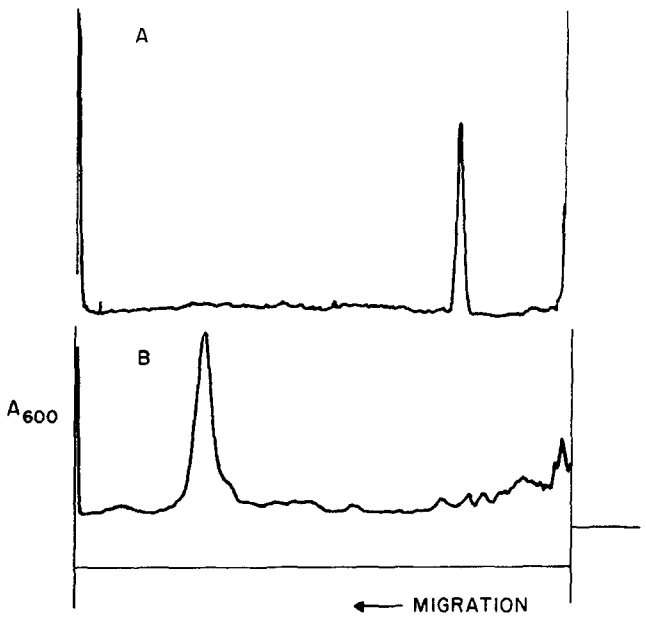

Figure 2 Densitometric tracing of SDS-Urea-polyacrylamide gels of rat liver OSCP (pooled fractions 35 to 50 from Figure 1) (A); and F_1-ATPase binding protein (pooled fractions 15 to 25) (B). Electrophoresis was performed according to the procedure of Swank and Munkres (15). Coomassie Blue was used for staining. The amount of protein applied was approximately 20 µg (A) and 45 µg (B).

Table I

Molecular weights and pI's of OSCP and the ATPase binding protein from rat liver mitochondria

Molecular weight	OSCP	Binding Protein
SDS + Urea gels (15)	23,500	8,500
SDS gel (samples treated with 1% BME)	22,500	8,500
SDS gel (without 1% BME treatment)	22,500	16,000
Isoelectric Point	9.5	9.1

two peaks, both with high oligomycin conferral activity. When these two peaks were analysed by SDS-gel electrophoresis, they both appeared as homogeneous single protein bands indistinguishable from each other (the mixture of the two migrated as a single band). We believe both peaks are OSCP, but one peak may be in a polymer form, similar to multiple forms of bovine heart OSCP (21). We are presently using sedimentation equilibrium method to determine the molecular weights of the two forms. In addition, fractions which eluted earlier (Fractions 15 to 25 in Figure 1) contained a protein with no OSCP activity, but assay results indicated that when these fractions were incubated with TUA particles and F_1, considerably higher amounts of F_1 were bound to the TUA particles (see Table II). When these fractions were pooled, precipitated by ammonium sulfate, and samples run in SDS-Urea gels, a major protein was seen with a molecular weight of 8,500, whereas the fractions containing OSCP gave a molecular weight of 22,500. Figure 2 shows the densitometric tracing of SDS-Urea gels of the purified OSCP (mixture of both active OSCP peaks) and the pooled Fractions 15 to 25. There were no observable contaminating bands in the OSCP gel, although several very minor bands were

Table II

Sensitivity of TUA particle-bound ATPase to Oligomycin in the presence of OSCP and the binding protein from rat liver mitochondria

	Addition of 200 µg of rat liver TUA particles		ATPase activity of particles (µmoles Pi/10 min)			ATPase activity of supernatants (µmoles Pi/10 min)	
	First[+] Incubation	Second[++] Incubation	- oligomycin	+ oligomycin	% Inhibition	First	Second
Experiment 1	None	omit	0.00	0.00	---	0.00	omit
	F_1, 5 µg	omit	0.90	0.90	0	0.12	omit
	F_1, 10 µg	omit	1.17	1.16	1.0	0.90	omit
Experiment 2	F_1, 5 µg Binding Protein 10 µg	omit	1.09	1.06	2.7	0.00	omit
	F_1, 10 µg Binding Protein 10 µg	omit	1.66	1.61	3.0	0.46	omit
	F_1, 10 µg Binding Protein 20 µg	omit	2.04	2.01	1.5	0.05	omit
Experiment 3	F_1, 10 µg	OSCP, 2 µg	1.16	0.51	56.0	0.84	0.00
Experiment 4	OSCP, 2 µg	F_1, 10 µg	1.20	0.62	48.3	omit	0.82
	OSCP, 4 µg	F_1, 10 µg	1.22	0.30	70.0	omit	0.79
	F_1, 10 µg OSCP, 2 µg	omit	1.23	0.53	57.1	0.80	omit
Experiment 5	F_1, 10 µg Binding Protein 10 µg + OSCP, 2 µg	omit	1.66	0.71	57.0	0.49	omit

+ TUA particles were incubated in 0.1 ml Tris-SO_4, pH 7.4 for 10 min at room temperature with the indicated components. After microcentrifugation, the supernatant and either the particles were assayed for ATPase activity (pellet with and without oligomycin , see Materials and Methods), or the pellet was used for second incubation.

++ The particles from first incubation mixture were resuspended in 0.1 ml of 0.1 M Tris-SO_4, with the additional added component and incubated at room temperature for 10 min. After microcentrifugation the supernatant and particles were assayed for ATPase activity (pellet with and without oligomycin).

visible in the high molecular weight regions for the combined Fractions 15 to 25.

The molecular weights were confirmed by running several different gel systems. Table I summarizes the results. The pure OSCP has a molecular weight of 22,000 to 23,000 both in SDS gel and SDS-Urea gel. The value obtained for bovine heart OSCP in our laboratory was also 22,000. In the gel systems that we used, the two OSCP's are indistinguishable. Interestingly, when pooled Fractions 15 to 25 were first incubated with 1% BME and analysed by SDS gel, the molecular weight was 8,500, in agreement with the result obtained by SDS-Urea gel system of Swank and Munkres (16) in which the samples were also preincubated in 1% DDT. However, when the binding

protein was analysed by SDS gel electrophoresis without preincubation with 1% BME, the molecular weight was 16,000. It seems that the 16,000 molecular weight species is a dimer of the species observed in the BME-treated sample. Possibly disulfide bonds may be involved in the formation of the dimer. The isoelectric points in urea gels of the purified rat liver OSCP and the F_1-ATPase binding protein were determined to be 9.5 and 9.1 respectively. The results are consistent with the elution patterns of the two proteins on the cation-exchanger column as shown in Figure 1.

The properties of the purified OSCP and the binding protein were examined and the results are shown in Table II. As indicated in Experiment 1, TUA particles were capable of binding some F_1-ATPase with the resultant membrane bound ATPase activity being insensitive to oligomycin; these results were similar to those obtained using the bovine heart mitochondrial system (22). However, the addition of the binding protein in the preincubation system resulted (Experiment 2) in additional F_1-ATPase binding to the TUA particles. Together with the observation that the resultant membrane bound ATPase activity was not inhibited by oligomycin this clearly indicates that the linking protein connecting the F_1-ATPase and the membrane is not OSCP. In addition, the results in Experiment 3 demonstrate that the binding site of OSCP in the membrane ATPase complex is somewhere other than the "stalk", since incubating pure OSCP with the membrane-bound ATPase renders the activity oligomycin-sensitive. It is confirmed further by the results of Experiment 4 that preincubating pure OSCP alone with TUA particles first, or preincubating OSCP, F_1-ATPase and TUA particles all together, only rendered the membrane-bound ATPase activity sensitive to oligomycin but did not increase the amount of F_1-ATPase bound by TUA particles. In contrast, as shown in Experiment 5, when both OSCP and the binding protein were preincubated with F_1-ATPase and TUA particles together, there was both an increased amount of F_1-ATPase bound by the particles and the resultant ATPase activity was sensitive to oligomycin.

The amino acid composition of rat liver OSCP is shown in Table III. It is very similar, but not identical to, that of bovine heart OSCP (2). Preliminary

Table III

Amino Acid Composition of OSCP from Rat Liver Mitochondria

	Residues/100 recovered residues
Aspartic Acid	8.27
Threonine †	4.39
Serine †	9.89
Glutamic Acid	14.21
Proline	4.42
Glycine	12.93
Alanine	8.85
Half Cystine ††	0.89
Valine	5.17
Methionine	1.65
Isoleucine	4.39
Leucine	7.43
Tyrosine	1.23
Phenylalanine	2.67

† 24-hour 6N HCl hydrolysis at 110°C; serine increased by 10% and threonine increased by 5% to compensate for destruction by acid.

††Performic acid-oxidized prior to acid hydrolysis; calculated from cysteic acid/alanine ratio.

results of amino acid composition of the binding protein (not shown) demonstrate that it is quite different from that of rat liver and bovine heart OSCP's.

In conclusion, we believe the results reported in this communication argue against the hypothesis that OSCP is the linking "stalk" connecting the F_1-ATPase to the membrane. The newly isolated binding protein, clearly different from OSCP, is responsible for binding the F_1-ATPase to the membrane. If indeed there is a stalk in in vivo mitochondria, as revealed by electron micrographs of negatively stained samples, it is more likely that this binding protein may either be the stalk or part of the stalk. We believe the previous assumption that OSCP is the connecting "stalk" resulted from two uncertainties. First, in reconstitution experiments using the bovine heart mitochondrial system, the F_1-ATPase binding activity of the TUA particles may be due to residual OSCP left in the particles. This possibility was not ruled out, since it is possible that oligomycin sensitivity conferral activity of

residual OSCP was lost, but the F_1-ATPase binding capability of OSCP was differentially maintained due to the very procedures of preparing TUA particles. Secondly, OSCP preparations used in many experiments (22) were not pure; thus, they might have contained the separate binding protein. In this report, we demonstrate that the binding of F_1-ATPase to the membrane is not due to OSCP, but is dependent on a separate protein with a molecular weight of 8,500 as determined by urea gel or SDS gel electrophoresis. In view of the present report, it would be of interest to ascertain whether the recently reported rat liver oligomycin-sensitive ATPase complex (23) contains this protein. It is also of great interest to compare this binding protein from rat liver to Fc_2 or F_6 from bovine heart mitochondria, since Fc_2 was originally isolated from bovine heart TUA particles and F_6 has been recently purified in apparent homogeneous form with a molecular weight of 8,000 (24). Preliminary results in our laboratory also indicate that rat liver OSCP is interchangeable with bovine heart OSCP (25), including the cross-reactivity of the former with the antibodies prepared against the latter, and substituting the rat liver OSCP in stimulation of ATP-^{32}Pi exchange activity in partially depleted heart mitochondrial particles. Thus, it seems that the conclusion in this report holds not only for rat liver mitochondria but may also be true for bovine heart mitochondria.

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References

1. MacLennan, D. H. and Tzagoloff, A. (1968) Biochemistry 7, 1603-1610.
2. Senior, A. E. (1971) J. Bioenergetics 2, 141-150.
3. Fessenden-Raden, J. M., Lange, A. J., Dannenberg, M. A. and Racker, E. (1969) J. Biol. Chem., 244, 6656-661.
4. Chan, S. H. P. (1971) Fed. Proc. 30, No. 3, Part II, Abstr. 1133.
5. Kagawa, Y. and Racker, E. (1966) J. Biol. Chem., 241, 2475-2482.
6. Senior, A. E. (1973) Biochim. Biophys. Acta, 301, 249-277.
7. Pedersen, P. L. (1975) Bioenergetics 6, 243-275.
8. Lehninger, A. L. (1975) Biochemistry, 2nd Edition, Worth Publishers, New York, p. 523.
9. Johnson, D. and Lardy, H. A. (1967) in Methods of Enzymology X, pp. 94-96, Ed. Pullman, M. E. and Estabrook, R. W. Academic Press.
10. Lambeth, D. O. and Lardy, H. A. (1971) Eur. J. Biochem., 22, 355-363.
11. Datta, A. and Penefsky, H. S. (1970) J. Biol. Chem., 245, 1537-1544.
12. Bulos, B. and Racker, E. (1968) J. Biol. Chem., 243, 3891-3900.
13. Pullman, M. E., Penefsky, H. A., Datta, A. and Racker, E. (1960) J. Biol. Chem., 235, 3322-3329.

14. Lohmann, K. and Jendrassik, L. (1926) Biochem. Z., 178, 419-426.
15. Weber, K. and Osborn, M. (1969) J. Biol. Chem., 244, 4406-4412.
16. Swank, R. T. and Munkres, K. D. (1971) Anal. Biochem., 39, 462-477.
17. Vesterberg, O. (1971) in Methods of Enzymology XXII, pp. 389-412, ed. Jakoby, W. B., Academic Press.
18. Gornall, A. G., Bardawill, C. J. and David, M. M. (1949) J. Biol. Chem., 177, 751.
19. Lowry, O. H., Rosenbrough, N. J., Farr, A. L. and Randall, R. J. (1951) J. Biol. Chem., 193, 265.
20. Spackman, D. H., Moore, S. and Stein, W. H. (1958) Anal. Chem., 30, 1190-1206
21. Chan, S. H. P. (1973) Fed. Proc. 32, 670.
22. Knowles, A. F., Guillory, R. J. and Racker, E. (1971) J. Biol. Chem., 246, 2672-2679.
23. Soper, J. W. and Pedersen, P. L. (1976) Biochemistry, 15, 2682-2690.
24. Kanner, B.I., Serrano, R., Kandrach, M. A. and Racker, E. (1976) Biochem. Biophys. Res. Commun. 69, 1050-1056.
25. Kleyman, T. R. and Chan, S. H. P. (1974) Fed. Proc. 33, 1920.