The above results demonstrate quite clearly that the N-5 position is crucial in order for the protein to be able to stabilize the half-reduced form of the flavin coenzyme. This is particularly emphasized by the fact that whereas 3-methyl-FMN and isoFMN bind to the Shethna apoprotein with approximately the same strength as does 5-deazaFMN (Edmondson and Tollin, 1971b), both of these analog proteins generate semiquinones upon either dithionite reduction or photoreduction (Edmondson and Tollin, 1971c). Although the deazaflavin radical is somewhat less stable than the normal flavin species (as judged by the faster disproportionation rate), this does not seem to be sufficient to account for the complete lack of semiquinone formation in the analog protein. The slower binding rates for the deaza analogs suggest that an important interaction occurs with the protein at the 5 position of the flavin ring. It is possible that this same interaction also participates in radical stabilization.

References

Beinert, H. (1960), *Enzymes 2*, 381.

D'Anna, Jr., J. A., and Tollin, G. (1971), *Biochemistry 10*, 57.

Dixon, M. (1971), *Biochim. Biophys. Acta 226*, 259.

Edmondson, D. E., and Tollin, G. (1971a), *Biochemistry 10*, 113.

Edmondson, D. E., and Tollin, G. (1971b), *Biochemistry 10*, 124.

Edmondson, D. E., and Tollin, G. (1971c), *Biochemistry 10*, 133.

Fleischman, D. E., and Tollin, G. (1965), *Proc. Nat. Acad. Sci. U. S. 53*, 23.

Flexser, L. A., and Farkas, W. G. (1952), U. S. Patent 2,610,179; *Chem. Abstr. 47*, 8781*g*.

Foust, G. P., Burleigh, Jr., B. D., Mayhew, S. G., Williams, Jr., C. H., and Massey, V. (1969), *Anal. Biochem. 27*, 530.

Gibson, Q. H., and Hastings, J. W. (1962), *Biochem. J. 83*, 368.

Gibson, Q. H., Massey, V., and Atherton, N. M. (1962), *Biochem. J. 85*, 369.

Hinkson, J. W., and Bulen, W. A. (1967), *J. Biol. Chem. 242*, 3345.

Holmström, B. (1964), *Acta Phys. Polon. 26*, 419.

Kierkegaard, P., Norrestam, R., Werner, P. E., Csöregh, I., Glehn, M. V., Karlsson, R., Leijonmarck, M., Rönnquist, O., Stensland, B., Tillberg, O., and Torbjörnsson, L. (1971), *in* Flavins and Flavoproteins, Kamin, H., Ed., Baltimore, Md., University Park Press, p 1.

Knowles, A., and Roe, E. M. F. (1968), *Photochem. Photobiol. 7*, 421.

Land, E. J., and Swallow, A. J. (1969), *Biochemistry 8*, 2117.

Massey, V., Müller, F., Feldberg, R., Schuman, M., Sullivan, P. A., Howell, L. G., Mayhew, S. G., Mathews, R. G., and Foust, G. P. (1969), *J. Biol. Chem. 244*, 3999.

Müller, F., Hemmerich, P., Ehrenberg, A., Palmer, G., and Massey, V. (1970), *Eur. J. Biochem. 14*, 185.

Müller, F., and Massey, V. (1969), *J. Biol. Chem. 244*, 4007.

O'Brien, D. E., Weinstock, L. T., and Cheng, C. C. (1970), *J. Heterocycl. Chem. 7*, 99.

Tollin, G. (1968), *Biochemistry 7*, 1720.

Tollin, G., and Edmondson, D. E. (1971), *in* Flavins and Flavoproteins, Kamin, H., Ed., Baltimore, Md., University Park Press, p 153.

Vaish, S. P., and Tollin, G. (1970), *J. Bioenerg. 1*, 181.

Vaish, S. P., and Tollin, G. (1971), *J. Bioenerg. 2*, 33.

A Comparison of the Nucleotide Specificity and Atractyloside Sensitivity of Digitonin and Sonic Particles†

David C. Hohnadel‡ and Cecil Cooper*

ABSTRACT: The specificity for phosphate acceptor of submitochondrial particles prepared by sonication and digitonin treatment was compared by use of a series of natural and synthetic analogs of ADP. The analogs examined contained changes in the purine ring, ribose, and the glycosidic linkage. The apparent K_m and V_{max} was determined for all analogs with both preparations. Contrary to previous reports, both preparations show broad specificity. The reason that digitonin particles appeared to be specific in earlier studies is that tests were conducted at a single high concentration of nucleotides at which GDP and IDP show marked substrate inhibition. The phosphate uptake with IDP as acceptor using digitonin particles was found to be inhibited by atractyloside. Two of the analogs were found to be substrates for nucleoside triphosphate synthesis with digitonin but not with sonic particles. These findings are at variance with current models of mitochondrial organization particularly with regard to the role of nucleotide translocase and the relative orientation of the vesicular membrane in digitonin and sonic particles.

Current theories on mitochondrial organization hold that ADP and ATP enter and leave the matrix compartment *via* an atractyloside-sensitive, adenine nucleotide specific translocase that is located in the inner membrane and catalyzes an exchange diffusion (Klingenberg and Pfaff, 1966; Heldt, 1967).

† From the Department of Biochemistry, Case Western Reserve University, Cleveland, Ohio 44106. *Received October 21, 1971.* This work was supported by a research grant (GB-12747) from the National Science Foundation and a training grant (GM-00035) from the National Institutes of Health.

‡ A portion of the data is from the thesis presented by D. C. H. in partial fulfillment of the requirements for the Ph.D. degree from Case Western Reserve University. Present address: Bristol Hospital, Bristol, Conn.

This concept arose from a variety of studies (Klingenberg, 1970) but some of the important evidence is based on observations in which the properties of various submitochondrial preparations are compared to those of intact mitochondria.

It has been reported that digitonin particles are similar to whole mitochondria in many ways while sonic particles are different. Some of these differences are as follows. (a) Proton translocation coupled to respiration is outward in intact mitochondria and digitonin particles and inward in sonic particles (Mitchell, 1966; Mitchell and Moyle, 1965); (b) oxidative phosphorylation and related exchange reactions are inhibited by atractyloside in intact mitochondria and digitonin particles but not in sonic particles (Bruni, 1966; Low *et al.*, 1963; Hoppel and Cooper, 1969b); (c) oxidative phosphorylation is specific for ADP as phosphate acceptor with both mitochondria and digitonin particles but not with sonic particles (Low *et al.*, 1963; Hoppel and Cooper, 1969b); (d) in reversed electron transport digitonin particles oxidize added cytochrome *c* and reduce only endogenous NAD whereas sonic particles oxidize only endogenous cytochrome *c* and reduce only exogenous NAD (Chance and Fugmann, 1961; Lee, 1963); (e) succinate oxidation by cytochrome *c* deficient digitonin particles can be restored by exogenous cytochrome *c* whereas succinate oxidation by cytochrome *c* deficient sonic particles requires both cytochrome *c* plus Triton X-100 (Tyler, 1970); (f) there is an energy-linked uptake of Ca^{2+} by mitochondria and digitonin particles but not by sonic particles (Vasington, 1963). These differences are presumed to result from the orientation of the membrane and this has been offered as an explanation of the differences between these preparations cited in items b and c above (Mitchell, 1966; Lee and Ernster, 1966; Malviya *et al.*, 1968; Heldt *et al.*, 1965; Pfaff *et al.*, 1965; Slater, 1966; Bygrave and Lehninger, 1967). The model is shown in Figure 1 and the following should be noted. In the digitonin particles (Figure 1A) the orientation of the inner membrane is thought to have been preserved so that the original matrix surface (stippled side) is in the interior of the vesicle and the availability of phosphate acceptor for the nonspecific phosphorylation (Low *et al.*, 1963) is still governed by the action of the adenine nucleotide specific nucleotide translocase (Pfaff and Klingenberg, 1968; Klingenberg, 1970). In contrast, the membrane of the sonic preparation is believed to have been everted by the preparative procedure and the ATP-synthesizing system is now in contact with the medium and independent of the action of nucleotide translocase (Figure 1B). This line of evidence then in favor of the model rests on the difference in atractyloside sensitivity and phosphate-acceptor specificity between intact mitochondria and digitonin particles on the one hand and sonic particles on the other.

Another explanation of these experimental data is that sonication affects the phosphorylation enzymes *per se* instead of providing a bypass of a nucleotide translocase enzyme. The sonicated preparation has a requirement for added divalent cation and its lack of specificity may arise from alterations in the binding and/or catalytic site produced by a loss of bound divalent cation rather than from an intrinsic lack of specificity of the phosphorylating enzyme. It has been recently shown by Hoppel and Cooper (1969b) that the nucleotide specificity of this preparation undergoes considerable variation when the concentration of added divalent cation is altered. IDP may have as little as 1.4% to as much as 26.5% of the activity of ADP depending on the experimental conditions.

We have attempted to further examine this latter possibility by more rigorously defining the phosphate-acceptor specificities of both digitonin and sonic preparations. To this end we have prepared a series of analogs of ADP containing modifications in the adenine ring, in the sugar, and in the base–sugar linkage. The suitability of these analogs to function as phosphate acceptors in oxidative phosphorylation has been examined by measuring the apparent K_m and V_{max} values for each of the compounds and comparing the results to those obtained with ADP.

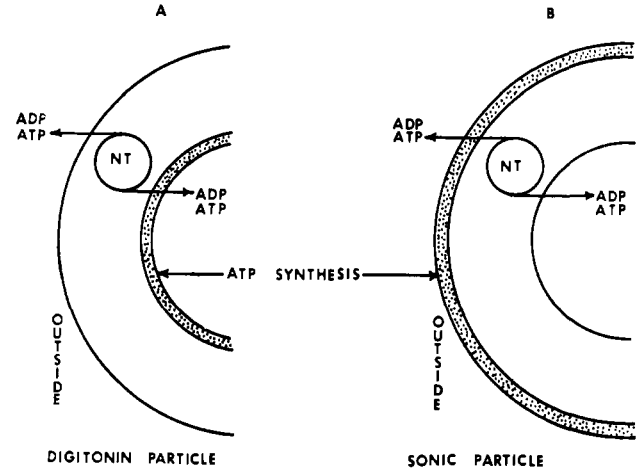

FIGURE 1: Relationship between membrane orientation and nucleotide specificity of sonic and digitonin submitochondrial particles. N. T. = atractyloside-sensitive, adenine nucleotide specific nuleotide translocase.

We wished to learn whether digitonin particles are absolutely specific for ADP and whether there are analogs of ADP capable of serving as phosphate acceptors with digitonin particles (presumed to require the translocase to make the nucleotides available to the phosphorylating enzymes) but not with sonic particles (no translocase needed). Such a finding would indicate that the model (Figure 1) as stated above may need revision.

Experimental Section

Rat liver mitochondria were isolated and the outer membrane removed by treatment with digitonin as described by Hoppel and Cooper (1968). The resulting inner membrane–matrix complex was then disrupted either by sonication or by treatment with digitonin to yield the B_{48} preparation previously described (Hoppel and Cooper, 1969a). The stock preparation containing 30–40 mg of submitochondrial particle protein/ml in 15% dimethyl sulfoxide–1 mM EDTA (pH 7.0) was stored at −80°.

The assay system for oxidative phosphorylation contained 20 mM 3-hydroxybutyrate, 20 mM morpholinopropanesulfonic acid, 10.0 mM inorganic phosphate containing 7–10 × 10^5 cpm of [^{32}P]P_i, 10 mM glucose, 1.0 mM $MgSO_4$, 0.25 mM NAD^+, varying amounts of nucleoside diphosphate, 20 EU of hexokinase, and 0.20 mg of particle protein in a final volume of 200 μl. The pH was 6.9 and the incubations were for 10 min at 30°. The formation of glucose 6-phosphate was followed by the extraction procedure previously described (Walters and Cooper, 1965). Phosphate uptake was linear over the incubation period with both particle preparations using K_m concentrations of all compounds tested.

The experimental data were corrected for the phosphate uptake observed (5–15 nmoles/10 min per 0.20 mg of protein)

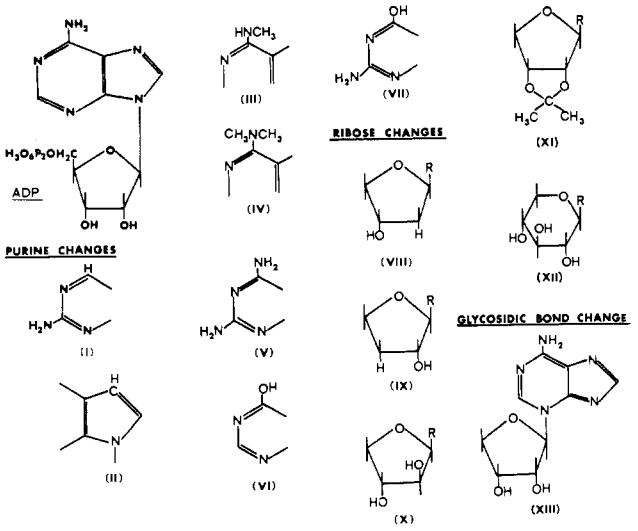

FIGURE 2: Structures of analogs tested. (I) 2-Aminopurine riboside 5′-diphosphate; (II) 7-deazaadenosine 5′-diphosphate; (III) N^6-monomethyladenosine 5′-diphosphate; (IV) N^6,N^6-dimethyladenosine 5′-diphosphate; (V) 2,6-diaminopurine riboside 5′-diphosphate; (VI) inosine 5′-diphosphate (IDP); (VII) guanosine 5′-diphosphate (GDP); (VIII) 2′dADP; (IX) 3′-dADP; (X) 9-(β-D-arabinofuranosyl)adenine 5′-diphosphate; (XI) 9-(β-D-isopropylidine ribofuranosyl)adenine 5′-diphosphate; (XII) 9-(β-D-glucpyranosyl)adenine 6′-diphosphate; (XIII) 3-(β-D-ribofuranosyl)-adenine 5′-diphosphate.

without added nucleoside diphosphate acceptor. Data from all experiments were calculated by an IBM 1620 computer in the $1/v$ *vs.* 1/(S) and (S)/v *vs.* (S) formats using the least-squares method for obtaining the best fit of the experimental points. The concentration ranges usually employed were 0.5–20 times the K_m value and in many cases the range was still larger.

The nucleotides were synthesized as described by Tener (1961) and Moffat (1964). The following modifications were introduced because difficulty was encountered in obtaining products free of extraneous phosphate. (1) The ratio of cyanoethyl phosphate to nucleoside was 1.5; (2) the cyanoethyl phosphate nucleoside diester was isolated by chromatography on QAE-Sephadex A-25 and elution with a linear gradient of 0–0.2 M triethylammonium bicarbonate (pH 7.5); (3) the isolated diester was hydrolyzed in 7 N NH_4OH at 100° for 15 min; (4) the products of the reaction between the morpholidate and pyrophosphate were separated on QAE-Sephadex A-25 using a gradient of 0–0.4 M triethylammonium bicarbonate (pH 9.2).

3-(β-D-Ribofuranosyl)adenine was synthesized by the method described by Leonard and Larsen (1965) and the 9-(β-D-glucopyranosyl)adenine by the method of Davoll and Lowry (1951). The structures of the analogs used are shown in Figure 2.

All nucleotides were converted to the K^+ salt by passage over Dowex 50. This was necessary because the triethylammonium bicarbonate was found to be inhibitory. All nucleotides were checked for purity by thin-layer chromatography on Kodak cellulose plates with isobutyric acid–concentrated NH_4OH–H_2O (66:33:1) and 1-butanol–H_2O–glacial acetic acid (5:3:2) as solvents. All showed a single spot under conditions in which a contamination of 0.1% could be detected (Randerath, 1967).

Hexokinase (type C-300) was obtained from Sigma. It was prepared for use by dialysis *vs.* 50 mM glucose–0.1 mM EDTA for 12 hr and was assayed as described by Darrow and Colowick (1962). 2-Aminopurine riboside, 2,6-diaminopurine riboside, and N^6-methyladenosine were purchased from Cyclo Chemical Co., N^6,N^6-dimethyladenosine from Sigma, and isopropylideneadenosine from Aldrich Chemical Co.

TABLE I: Ability of Analogs of ADP to Act as Acceptors in Oxidative Phosphorylation by Sonicated Particles.[a]

Preparation	Compound	K_m (× 10^{-6} M)	V_{max} (nmoles/min per mg)	K_m XDP/ K_m ADP	V_{max} XDP/ V_{max} ADP
A	ADP (10)	13.6 ± 2.3	257 ± 15	1.00	1.00
B	ADP (6)	11.0 ± 1.0	250 ± 2	1.00	1.00
A	I (2)	82.5 ± 4.5	15.8 ± 0.3	6.07	0.06
A	II (2)	10.0 ± 1.8	49.8 ± 1.5	0.74	0.19
A	III (2)	43.3 ± 3.0	122 ± 2	3.18	0.47
A	IV (2)	109 ± 21	98.7 ± 1.1	8.02	0.38
A	V (2)	100 ± 2	186 ± 1	7.35	0.72
A	VI (2)	322 ± 10	92 ± 0.6	23.7	0.36
A	VII (2)	577 ± 53	60 ± 1.4	41.0	0.23
A	VIII (4)	18.7 ± 0.8	92.2 ± 1.2	1.38	0.36
A	IX (2)	32.0 ± 1.0	228 ± 2	2.35	0.89
B	X (2)	201 ± 9	40.0 ± 0.4	18.3	0.16
A	XI (3)	Not a substrate			
A	XII (3)	Not a substrate			
A	XIII (2)	92.5 ± 5.0	85.8 ± 1.1	6.80	0.33

[a] The assay conditions are described in the text. The figures in parentheses give the number of determinations and the kinetic values are mean ± SD. A and B refer to different preparations of sonic particles and the ratios shown in columns 5 and 6 are compared to the values for ADP determined with the same preparation.

Structural Analogs of ADP as Phosphate Acceptors. Before the various nucleoside diphosphates can be tested as phosphate acceptors, it is necessary to make certain that the reaction velocity is not being determined by the activity of the hexokinase-trapping system. With ADP, equal values of K_m and V_{max} were obtained with 3–20 EU of hexokinase, and with 2′-dADP (VIII) 10 and 20 EU gave equal values but 3 EU gave a slightly lower V_{max}. In order to ensure that an excess was present during subsequent experiments 20 units of hexokinase was used per assay. Experiments with I, X, XI, and XII which are poor substrates (Tables I and II) were also carried out using 60 units of hexokinase/assay. Values of K_m and V_{max} equal to those obtained with 20 units of hexokinase testified that the coupling enzyme was not the rate-limiting component even with the poorest substrates.

The results obtained with the analogs were shown in Tables I and II. It should be noted that XI and XII are not substrates for the sonic preparation (Table I) but are for the digitonin preparation (Table II). Another point worthy of mention is that IDP (VI) and GDP (VII) are substrates for the digitonin particles although previous reports indicated they were not (Cooper and Lehninger, 1956; Hoppel and Cooper, 1969b).

Two questions must be considered in order to understand the significance of the data presented. These are as follows. (1) Why do the digitonin particles exhibit as broad a specificity as the sonic particles when previous reports indicated

TABLE II: Ability of Analogs of ADP to Act as Acceptors in Oxidative Phosphorylation by Digitonin Particles.[a]

Preparation	Compound	K_m ($\times 10^{-6}$ M)	V_{max} (nmoles/min per mg)	K_m XDP/ K_m ADP	V_{max} XDP/ V_{max} ADP
C	ADP (10)	2.0 ± 1.0	63.5 ± 1.8	1.00	1.00
D	ADP (6)	3.9 ± 1.0	112 ± 8	1.00	1.00
E	ADP (6)	2.7 ± 0.6	98.8 ± 3.7	1.00	1.00
C	I (2)	0.7 ± 1.6	11.5 ± 1.5	0.35	0.18
C	II (2)	3.6 ± 0.8	56.8 ± 3.9	1.80	0.89
C	III (2)	13.1 ± 4.0	37.7 ± 1.3	6.55	0.59
C	IV (2)	74.0 ± 9.2	10.3 ± 0.4	37.0	0.16
E	V (2)	1.8 ± 1.0	38.3 ± 0.4	0.67	0.39
D	VI (2)	17.1 ± 2.5	10.9 ± 0.3	4.38	0.10
E	VII (6)	5.9 ± 4.6	3.6 ± 0.2	2.19	0.04
C	VIII (4)	4.3 ± 0.9	49.6 ± 2.5	2.15	0.78
C	IX (4)	5.3 ± 1.2	64.4 ± 3.3	2.65	1.01
D	X (2)	4.0 ± 0.3	13.9 ± 0.7	1.03	0.12
D	XI (4)	10.3 ± 4.9	1.8 ± 0.1	2.64	0.02
D	XII (2)	32.5 ± 14	4.8 ± 0.4	8.33	0.04
D	XIII (2)	8.3 ± 3.3	28.8 ± 0.1	2.13	0.26

[a] The assay conditions are described in the text. The figures in parentheses give the number of determinations and the kinetic values are mean ± SD. C, D, and E refer to different preparations of digitonin particles and the ratios shown in columns 5 and 6 are compared to the values for ADP determined with the same preparations.

they are absolutely specific for ADP? (2) Are the sonic particles incapable of using XI and XII as substrates because they lack a binding site or because of the specificity of the catalytic site?

The answer to the first question is evident from a measurement of phosphate uptake as a function of nucleoside diphosphate concentration (Figure 3). In the cases where GDP and IDP are used as phosphate acceptor there is a pronounced substrate inhibition beginning at about 0.6–0.8 mM and this sharply increases until at 1.5–2.0 mM the phosphate uptake is completely lost. ADP used at levels up to 5.0 mM shows no signs of inhibition. The other specificity studies that have been done with digitonin particles used relatively high concentration of phosphate acceptor (2.5–5.0 mM) and they would therefore be expected to appear to be specific for ADP.

The answer to the second question is provided by the data shown in Figure 4 which show the effect of the presence of XI and XII on phosphate uptake with ADP as acceptor. Both XI (Figure 4A) and XII (Figure 4B) appear to compete with ADP for a binding site on the sonic particles. Secondary plots (not shown) made from these data indicate that the actual type of inhibition varies from competitive to mixed as the concentration of inhibitor is increased. Because of this it is difficult to obtain meaningful inhibitor constants. These experiments show that the two analogs can bind to the sonic particles but cannot be converted to the corresponding triphosphate.

Specificity of Inhibition of Atractyloside. The finding that digitonin particles are not specific for ADP as phosphate acceptor raises the interesting question of whether the inhibition of oxidative phosphorylation by atractyloside in these preparations is specific for the phosphate acceptor used. The model shown in Figure 1 dictates that only the uptake with ADP should be affected since only the ADP is arriving at the phosphorylation site *via* an adenine nucleotide specific and atractyloside-sensitive nucleotide translocase. The phosphorylation of the other nucleoside diphosphate could presumably arise *via* atractyloside-insensitive diffusion.

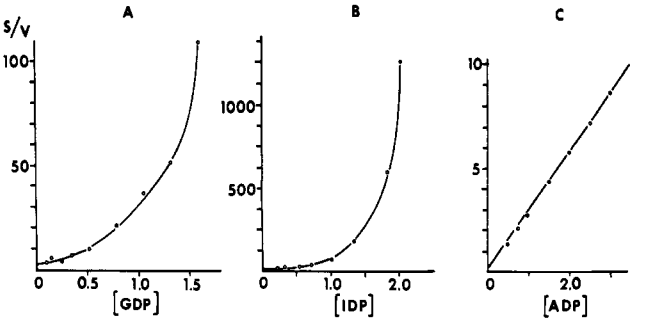

FIGURE 3: Effect of nucleotide concentration on phosphate uptake by digitonin particles. Velocity = nmoles/min per mg of protein; substrate concentration = mM.

The data for an experiment in which the atractyloside sensitivity using ADP, IDP (VI), and 7-deazaadenosine-5′-diphosphate (II) as phosphate acceptor is shown in Table III. Clearly, the uptake with all three acceptors is atractyloside sensitive.

Discussion

The findings reported above indicate that the model shown in Figure 1 is not tenable as stated. Compounds XI and XII cannot be used as substrates by the sonic particles even though they apparently are bound (see Figure 3). Since the specificity is thought to reside exclusively in the nucleotide translocase, it should not be possible to have a substrate function with digitonin particles (presumed to contain a functional translocase) and not with sonic particles where phosphorylation is not presumed to involve translocase. We have suggested that the properties of the binding and/or catalytic site on the phos-

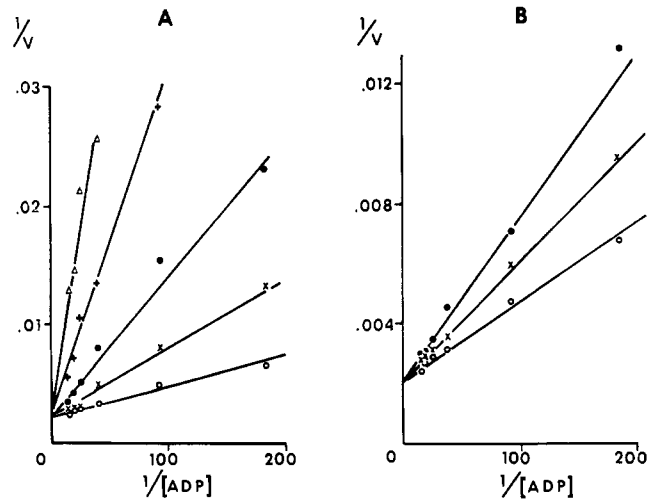

FIGURE 4: Effect of XI (A) and XII (B) on phosphate uptake with ADP by sonic particles (○) ADP alone; (×) ADP plus 1.43 μM analog; (●) ADP plus 4.83 μM analog; (+) ADP plus 16.3 μM analog; (△) ADP plus 57.4 μM analog. Velocity = nmoles/ min per mg of protein; ADP concentration = mM.

TABLE III: Inhibition by Atractyloside of Phosphate Uptake by Digitonin Particles with Different Phosphate Acceptors.[a]

Acceptor	Atractyloside Concn/ Acceptor Concn	% Inhibition
ADP	3.6	89
	12	91
	36	93
7-Deazaadenosine 5′-diphosphate (II)	2.9	89
	8.8	92
	36	94
IDP (VI)	0.5	78
	1.6	87
	17	100

[a] The assay conditions are described in the text. The concentration of atractyloside used was 50 μM.

phorylating enzyme is altered during the course of preparing the sonic particles (Hoppel and Cooper, 1969b). This means that the differences between sonic and digitonin particles may arise from an altered enzyme rather than from everting the inner membrane. A combination of both circumstances is, of course, also possible.

It is interesting to note that both preparations can use most of the compounds tested providing they are tested under appropriate conditions. Comparative tests made only at high concentrations of nucleotides yield misleading results. Data in the literature concerning relative specificities of enzymes for substrates, cofactors, metals, etc., are subject to the same criticism.

The observation that phosphate uptake with ADP as acceptor is atractyloside sensitive has not been previously reported and would not have been predicted from the model shown in Figure 1. There are at least three obvious explanations. The first is that the atractyloside-sensitive nucleotide translocase is not specific for adenine nucleotides. Studies in the literature indicate that the enzyme is specific for adenine nucleotides (Pfaff and Klingenberg, 1968; Klingenberg, 1970) but these appear to have been done at a single nucleotide concentration (10^{-4} M). It is not certain that the inactivity with other nucleotides may not have resulted from inhibition by excess substrate although this seems unlikely at that concentration. The second possibility is that the atractyloside sensitivity with IDP is apparent rather than real and arises from a fortuitous combination of conditions, namely, the presence of intravesicular ADP and extravesicular nucleoside diphosphokinase. This could result in reactions 1–4. The reentry of ADP_{out} (reaction 4) would be blocked by atractyloside and the overall conversion of IDP to ITP would appear to be sensitive. Both

$$ADP_{in} + P_i \longrightarrow ATP_{in} \quad (1)$$

$$ATP_{in} \longrightarrow ATP_{out} \quad (2)$$

$$IDP_{out} + ADP_{out} \longrightarrow ITP_{out} + ADP_{out} \quad (3)$$

$$ADP_{out} \longrightarrow ADP_{in} \quad (4)$$

the digitonin and sonic particles are prepared from mitochondria first treated to remove their outer membrane. The nucleoside diphosphokinase is believed to be located in the space between the inner and outer membrane (Schnaitman and Greenawalt, 1968) and would therefore be largely removed. These preparations have been previously shown to be very low in, but not completely devoid of, nucleoside diphosphokinase (Hoppel and Cooper, 1969a,b). As long as some of this enzyme remains, this explanation cannot be completely eliminated but its low activity makes it unlikely. The third explanation holds that the atractyloside sensitivity results from an effect at the nucleotide binding site of the phosphorylation enzyme rather than on a translocase. The lack of sensitivity of the sonic particles would arise from an alternation of the binding site by the preparative procedure. One major difference between the digitonin and sonic preparations is that the former shows no Mg^{2+} requirement whereas the latter has an absolute Mg^{2+} requirement. It is known that the atractyloside sensitivity can be overcome in a competitive fashion by the addition of adenine nucleotides (Bruni *et al.*, 1965; Hoppel and Cooper, 1969b). It is also known that Mg^{2+} plays an unexplained role in determining the degree of sensitivity to atractyloside. Digitonin particles are much more sensitive to atractyloside in the presence of Mg^{2+} (Hoppel and Cooper, 1969b). In addition, the nucleotide specificity for phosphate uptake by sonic preparations shows a marked alteration depending on the Mg^{2+} concentration. The ability of IDP to serve as phosphate acceptor may vary from 1.4 to 26.5% of the activity with ADP when the Mg^{2+} concentration is altered (Hoppel and Cooper, 1969b). This has led us to concur with the suggestion by Bruni (Bruni *et al.*, 1965; Bruni, 1966) that atractyloside acts directly on the phosphorylation enzyme and our current findings are readily explicable on this basis.

We were unable to show phosphate uptake with intact mitochondria when the IDP concentration was varied between 3 and 300 μM. It is possible that nucleotide translocase is functional in intact mitochondria but not in digitonin particles. However, it is also possible that the ATP synthesizing system is more "intact" and specific in whole mitochondria than in digitonin particles.

Under the conditions employed here the K_m for ADP with digitonin particles is 2–3 μM which is about the same as the K_m determined with intact mitochondria for oxidative phosphorylation (2–3 μM) and ADP translocation (4 μM) (Klingenberg and Pfaff, 1968; Klingenberg, 1970).

A comparison of the effect of structural alteration on the binding and catalytic activity of a number of analogs (Tables I and II) is of interest. The digitonin particles do not show a binding specificity dependent on the ribose portion of the molecule. Compounds like VIII, IX, X, XI, and XIII all bind almost as well as ADP. The ratio of K_m for XDP to K_m for ADP rises to a maximum of only 2.65 for these compounds. However, if the ribose is replaced by glucose a large increase in K_m results. The sonic particles on the other hand seem to show some involvement of the ribose in binding. Replacement of the ribose with arabinose produces a marked elevation of the K_m. Changing the position of the glycosidic bond (XIII) also increases the K_m substantially. Although XI and XII are not substrates they can act as inhibitors and therefore are probably capable of being bound. It is possible however that they can only be bound in the presence of ADP and cannot be bound to the existing "native" binding site.

The 6-amino group seems to be of considerable importance in determining the K_m for both digitonin and sonic particles. The K_m increases as the amino hydrogens are replaced by

methyl groups or if the amino group is replaced by a hydroxyl group as in IDP (VI). A further difference in structural specificity for binding between the two preparations is that the presence of a 2-amino group (I and V) decreases the K_m in digitonin particles but increases it in sonic particles. When a 2-amino group is combined with a 6-hydroxyl group as in GDP (VII) the effect on K_m is synergistic for sonic particles (82.5 and 322 *vs.* 557) but is approximately additive for digitonin particles (0.7 and 17.1 *vs.* 5.9).

An examination of the effects of structural alteration on *catalytic* activity also shows some interesting aspects. The presence of the 2′- and 3′-hydroxyls seem to be unimportant for the digitonin particles whereas the absence of the 2-hydroxyl produces a drop in V_{max} of 64% with the sonic particles. Even though the digitonin particles do not show a requirement for the 2′- or 3′-hydroxyls, the alteration of their cis configuration to the trans configuration found in arabinose (X) or the attachment of an isopropylidine group (XI) causes a marked drop in V_{max}. The loss of activity with the glucosyl compound (XIII) may be caused by the presence of trans rather than cis hydroxyls at positions 2′ and 3′ or steric restrictions in folding. The sonic particles also show a large drop in V_{max} with X and they are completely inactive with XI and XII.

The effect of changes in the purine ring on V_{max} are also of importance. As was the case with the K_m, the V_{max} is also dependent on the 6-amino group. If the hydrogens are replaced by methyl groups, the V_{max} is decreased. If it is replaced with a hydroxyl group (VI) there is also a marked drop. If the amino group is at position 2 instead of 6 the V_{max} is very low but if the 6-amino group is also present (V) then there is a smaller degree of inhibition. Nevertheless the 2-amino group itself seems to be somewhat inhibitory.

One other compound of considerable interest is II. In this case the N-7 has been replaced by a carbon atom. This position is involved in the formation of a backbound metal–ATP complex with Mn^{2+}, Co^{2+}, and Ni^{2+} (Glassman *et al.*, 1971; Kuntz *et al.*, 1972). If such a conformation is also important for the nucleoside diphosphate to act as a substrate *with* Mg^{2+} then large changes would be expected. The effect on K_m is small for both preparations but the sonic particles have a low V_{max} whereas the change in V_{max} with the digitonin particles is small.

Another group that causes a large change in V_{max} is moving the glycosidic bond from N-9 to N-3 (XIII). Both preparations are affected to about the same extent.

In general, the sonic particles appear to have a greater binding specificity for the ribose than do the digitonin particles. The 6-amino group is important for both preparations for both binding and catalysis and the presence of a 2-amino group seems to increase the affinity of the digitonin particles for the nucleotide.

It should be pointed out that the observed effects of structural alterations do not appear to be an indirect result of the ability of the nucleoside triphosphate to serve as a substrate for hexokinase *vs.* particle ATPase. First, the use of very high levels of hexokinase with the poorest substrates had no effect on K_m and V_{max}. We have also examined the specificity of the yeast hexokinase with the corresponding nucleoside triphosphate analogs and found that the activity with even the poorest hexokinase substrates, VII and IX, was more than adequate to keep pace with the rate of phosphate uptake.[1]

These findings, that the structural requirements for binding and catalytic activity differ in the digitonin and sonic preparations, are readily explained by the assumption that digitonin treatment and sonication have different effects on the phosphorylation enzyme *per se* rather than on the orientation of the inner mitochondrial membrane.

[1] D. C. Hohnadel and C. Cooper, unpublished results.

Acknowledgment

We would like to acknowledge the devoted and expert technical assistance of Mrs. Ksenja Dimitrov. We also thank Mr. Thomas A. Glassman for providing XII and XIII, Dr. T. H. Haskell of Parke-Davis Research Laboratories for 9-(β-D-arabinofuranosyl)adenosine, Dr. A. R. Hanze of Up-John for 7-deazaadenosine, and Dr. A. J. Guarino, Department of Biochemistry, University of Texas, San Antonio, Texas, for 3′-deoxyadenosine.

References

Bruni, A. (1966), *Biochim. Biophys. Acta Lib. 7*, 275.
Bruni, A., Luciani, S., and Bortignon, C. (1965), *Biochim. Biophys. Acta 97*, 434.
Bygrave, F. L., and Lehninger, A. L. (1967), *Proc. Nat. Acad. Sci. U. S. 57*, 1409.
Chance, B., and Fugmann, V. (1961), *Biochem. Biophys. Res. Commun. 4*, 317.
Cooper, C., and Lehninger, A. L. (1956), *J. Biol. Chem. 219*, 489.
Darrow, R. A., and Colowick, S. P. (1962), *Methods Enzymol. 5*, 226.
Davoll, J., and Lowry, B. A. (1951), *J. Amer. Chem. Soc. 73*, 1650.
Glassman, T. A., Cooper, C., Harrison, L. W., and Swift, T. J. (1971), *Biochemistry 10*, 843.
Heldt, H. W. (1967), *in* Mitochondrial Structure and Compartmentation, Quagliariello, E., Papa, S., Slater, E. C., and Tager, J. M., Ed., Bari, Adriatica Editrice, p 260.
Heldt, H. W., Jacobs, H., and Klingenberg, M. (1965), *Biochem. Biophys. Res. Commun. 18*, 174.
Hoppel, C., and Cooper, C. (1968), *Biochem. J. 107*, 367.
Hoppel, C., and Cooper, C. (1969a), *Arch. Biochem. Biophys. 135*, 173.
Hoppel, C., and Cooper, C. (1969b), *Arch. Biochem. Biophys. 135*, 184.
Klingenberg, M. (1970), *Essays Biochem. 6*, 119.
Klingenberg, M., and Pfaff, E. (1966), *Biochim. Biophys. Acta Lib. 7*, 180.
Klingenberg, M., and Pfaff, E. (1968), *Biochem. Soc. Symp. 27*, 105.
Kuntz, G. P. P., Glassman, T. A., Cooper, C., and Swift, T. J. (1972), *Biochemistry 11*, 538.
Lee, C. P. (1963), *Fed. Proc., Fed. Amer. Soc. Exp. Biol. 22*, 527.
Lee, C. P., and Ernster, L. (1966), *Biochim. Biophys. Acta Lib. 7*, 218.
Leonard, J. J., and Larsen, R. A. (1965), *Biochemistry 4*, 354.
Low, H., Vallin, I., and Alm, B. (1963), *in* Energy Linked Functions in Mitochondria, Chance, B., Ed., New York, N. Y., Academic Press, p 5.
Malviya, A. N., Parsa, B., Yodaiken, R. E., and Elliott, W. B. (1968), *Biochim. Biophys. Acta 162*, 195.
Mitchell, P. (1966), *Biol. Rev. 41*, 445.
Mitchell, P., and Moyle, J. (1965), *Nature (London) 208*, 1205.
Moffat, J. G. (1964), *Can. J. Chem. 42*, 599.
Pfaff, E., and Klingenberg, M. (1968), *Eur. J. Biochem. 6*, 66.

Pfaff, E., Klingenberg, M., and Heldt, H. W. (1965), *Biochim. Biophys. Acta 104*, 312.
Randerath, K. (1967), *Anal. Biochem. 21*, 480.
Schnaitman, C., and Greenawalt, J. W. (1968), *J. Cell Biol. 38*, 158.
Slater, E. C. (1966), *Comp. Biochem. 14*, 327.
Tener, G. M. (1961), *J. Amer. Chem. Soc. 83*, 149.
Tyler, D. D. (1970), *Biochem. J. 116*, 30P.
Vasington, F. D. (1963), *J. Biol. Chem. 238*, 1841.
Walters, E., and Cooper, C. (1965), *Anal. Biochem. 10*, 370.

Identification of the Dicyclohexylcarbodiimide-Binding Protein in the Oligomycin-Sensitive Adenosine Triphosphatase from Bovine Heart Mitochondria†

Feico S. Stekhoven,* Richard F. Waitkus, and Herman Th. B. van Moerkerk

ABSTRACT: Dicyclohexylcarbodiimide in concentrations not exceeding those necessary for maximal inhibition of ATPase activity binds specifically to a protein in an oligomycin- and carbodiimide-sensitive ATPase complex (OS-ATPase) isolated from bovine heart mitochondria. This protein, designated the carbodiimide-binding protein, has a molecular weight of 13,000–14,000. At concentrations exceeding the maximum inhibition level, the carbodiimide becomes bound also to other proteins, like F_1, present in the ATPase complex. Crude carbodiimide-binding protein can be extracted from OS-ATPase with chloroform–methanol (2:1, v/v) as a water-insoluble proteolipid.

One of the characteristics of the mitochondrial Mg^{2+}-dependent ATPase is its inhibition by oligomycin or dicyclohexylcarbodiimide (Lardy *et al.*, 1958, 1965; Huijing and Slater, 1961; Beechey *et al.*, 1967; Beyer *et al.*, 1967), both inhibitors having a similar mode of action (Beechey *et al.*, 1966, 1967; Racker and Horstman, 1967; Bulos and Racker, 1968a,b; Roberton *et al.*, 1968).

A soluble Mg^{2+}-dependent ATPase, F_1,[1] has been isolated from beef heart mitochondria as well as from yeast mitochondria (Pullman *et al.*, 1960; Schatz *et al.*, 1967; Tzagoloff, 1969a) but these preparations are not inhibited by oligomycin or the carbodiimide (Racker *et al.*, 1961; Racker, 1962, 1963; Bulos and Racker, 1968a; Schatz *et al.*, 1967; Tzagoloff, 1969b), except by high concentrations of the latter inhibitor (Penefsky, 1967) exceeding those necessary for blocking particulate ATPase activity (Beechey *et al.*, 1967; Beyer *et al.*, 1967; Bulos and Racker, 1968a). In addition submitochondrial particles have been isolated, depleted in F_1, and containing only residual ATPase activity. Addition of F_1 to these particles restored particle-bound ATPase activity which is then oligomycin sensitive (Racker *et al.*, 1961; Racker, 1962, 1963; Kagawa and Racker, 1966a). Subsequently it was shown by studies with radioactive rutamycin, an oligomycin analog (Thompson *et al.*, 1961; Lardy *et al.*, 1965) that the F_1-deficient particles are the site of action of the inhibitor (Kagawa and Racker, 1966b).

Later more depleted submitochondrial particulate preparations have been isolated which require different soluble protein factors like oligomycin-sensitivity-conferring protein (OSCP) (MacLennan and Tzagoloff, 1968; Tzagoloff, 1970), also called Fc or Fc_1 (Bulos and Racker, 1968a,b; Knowles *et al.*, 1971), and Fc_2 (Knowles *et al.*, 1971) to confer oligomycin or the carbodiimide sensitivity to F_1-ATPase. Yet neither one of these factors appears to bear the site of oligomycin or the carbodiimide inhibition, as this was found to be located in the particles depleted of the complementary factors used in the assay. It was furthermore found that these particles lose their ability to confer oligomycin or the carbodiimide sensitivity to F_1 after heat or trypsin treatment, indicating that they contain still another protein necessary for reconstitution of the oligomycin-sensitive ATPase (Bulos and Racker, 1968a,b; Knowles *et al.*, 1971).

It is the aim of this article to pinpoint the site of action of the carbodiimide by use of the radioactive chemical as suggested by Bulos and Racker (1968a). This approach is feasible, since in contrast to oligomycin (Kagawa and Racker, 1966b; Bulos and Racker, 1968a) the carbodiimide is irreversibly bound to the particulate preparations as shown by the fact that inhibition of ATPase cannot be released by washing with phospholipids (Holloway *et al.*, 1966; Bulos and Racker, 1968a). Since we felt that the heat- and trypsin-sensitive component in the depleted particles mentioned above could be the carbodiimide-binding protein, we have concentrated our

† From the Department of Biochemistry, University of Nijmegen, Medical School, Geert Grooteplein Noord 21, Nijmegen, The Netherlands. *Received October 25, 1971.* This investigation was supported in part by the Netherlands Organization for Basic Research (Z. W. O.).

[1] Abbreviations used that are not listed in *Biochemistry 5*, 1445 (1966), are: F_1, coupling factor F_1 (ATPase) (Pullman *et al.*, 1960); OSCP, oligomycin-sensitivity-conferring protein (MacLennan and Tzagoloff, 1968); Fc (Fc_1), rutamycin- (or dicyclohexylcarbodiimide-) sensitivity factor (Bulos and Racker, 1968a,b; Knowles *et al.*, 1971); Fc_2, factor conferring the dicyclohexylcarbodiimide sensitivity to F_1 in presence of Fc_1 and TUA-STA particles (Knowles *et al.*, 1971); OS-ATPase, oligomycin-sensitive ATPase complex isolated from bovine heart mitochondria by the procedure of Tzagoloff *et al.* (1968a); TUA particles, trypsin–urea-treated submitochondrial particles which were sonicated in the presence of ammonia (Bulos and Racker, 1968a); TUA-CF_o, cholate-treated TUA particles (Bulos and Racker, 1968b); CF_o, trypsin–urea–cholate-treated submitochondrial particles (Kagawa and Racker, 1966b); TUA-STA, silicotungstate-treated TUA particles (Knowles and Guillory, 1970; Knowles *et al.*, 1971).