Biochimica et Biophysica Acta, 589 (1980) 1—9

BBA 47774

BIOLOGICAL ACTIVITY OF SYNTHETIC MOLYBDENUM-IRON-SULPHUR, IRON-SULPHUR AND IRON-SELENIUM ANALOGUES OF FERREDOXIN-TYPE CENTRES

MICHAEL W.W. ADAMS [a], K. KRISHNA RAO [a], DAVID O. HALL [a,*]
GEORGE CHRISTOU [b] and C. DAVID GARNER [b]

[a] *Department of Plant Sciences, King's College University of London, 68 Half Moon Lane, London SE24 9JF (U.K.) and* [b] *Chemistry Department, Manchester University, Manchester (U.K.)*

(Received May 21st, 1979)

Key words: Ferredoxin analog; Fe-S cluster; Fe-Se cluster; Mo-Fe-S cluster; Nitrogenase; Hydrogenase

Summary

The molybdenum-iron-sulphur cluster $[Fe_6Mo_2S_8(SCH_2CH_2OH)_9]^{3-}$, which contains two Fe_3MoS_4 cubane-like centres, is the best plausible analogue available to date for the molybdenum site of the nitrogenase enzymes. The iron-sulphur cluster $[Fe_4S_4(S \cdot CH_2CH_2OH)_4]^{2-}$ and the iron-selenium cluster $[Fe_4Se_4(S \cdot CH_2CH_2OH)_4]^{2-}$ are structural analogues of the ferredoxin Fe_4S_4 active centre. All three clusters would replace ferredoxin and mediate electron transfer to *Clostridium pasteurianum* hydrogenase in a H_2-evolving system with sodium dithionite as the electron donor. The clusters would not replace hydrogenases which themselves are unable to evolve H_2 from reduced ferredoxins. The molybdenum-iron-sulphur cluster would also replace ferredoxin in a chloroplast-ferredoxin-hydrogenase H_2 evolving system.

Introduction

Recent extended X-ray absorption fine structure (EXAFS) studies on the nitrogenases from *Azotobacter vinelandii* and *Clostridium pasteurianum* and on the FeMo-cofactor of the former [1,2] have suggested that a possible structural candidate for this molybdenum site is an $[Fe_3MoS_4]$ cubane-like system. These data led us to investigate the reaction chemistry of mixtures

* To whom all correspondence should be addressed.
Abbreviation: Me_2SO, dimethyl sulfoxide.

containing molybdenum, iron and sulphur, and subsequently to synthesise and structurally characterise complexes (I) containing two $[Fe_3MoS_4]$ units bridged across their molybdenum atoms by three μ-thiolato-groups [3,4].

$[NR_4]_3[Fe_6Mo_2S_8(SR)_9]$ (R = Ph,Et, etc.)

(I)

Although the mechanism of nitrogenase action has still to be satisfactorily elucidated, the molybdenum site and presumably, therefore, the Fe_3MoS_4 unit can be assumed (at least) to be involved in electron-transfer processes involving substrate and/or another iron-sulphur centre. Recently cyclic voltammetric [5] and differential pulse polarographic measurements (Christou, G., Garner, D. and Miller, R.M., unpublished data) in Me_2SO have shown that complexes (I) are capable of undergoing two reversible one-electron reductions. As an initial enquiry into the electron-transfer properties of these clusters we have investigated their ability to mediate electron-transfer to the hydrogenase enzyme (H_2:ferredoxin oxidoreductase, EC 1.12.7.1), in hydrogen-evolution systems, using dithionite or illuminated chloroplasts as the electron donor. The experiments were performed in aqueous solution to prevent denaturation of the enzyme and the water-soluble complex (I; R = Et, R′ = CH_2CH_2OH; see Ref. 4) was employed. The earlier studies [6] of electron transfer reactions of the ferredoxin active-site analogue $(NMe_4]_2[Fe_4S[SCH_2CH_2OH)_4]$ and its selenium homologue $(NMe_4]_2[Fe_4Se_4(SCHCH_2OH)_4]$ have been extended.

Materials and Methods

All chemicals were of the highest available purity and were obtained from BDH Chemicals, Ltd., Poole, Dorset, U.K. unless otherwise stated. Preparation of the analogue clusters: $[NEt_4]_3[Fe_6Mo_2S_8(SCH_2CH_2OH)_9]Me_2CO$ was prepared by ligand exchange as previously reported [4]. $[NMe_4]_2[Fe_4S_4(SCH_2CH_2OH)_4]$ was prepared from elemental sulphur and $[NMe_4]_2[Fe_4Se_4(SCH_2CH_2OH)_4]$ from elemental selenium using the procedure reported for the sulphur complex [7]. Stock solutions were prepared in either Me_2SO or 50 mM Tris-HCl buffer, pH 8.5; the latter solutions also contained five equivalents of mercaptoethanol per ligated $HOCH_2CH_2S^-$ to prevent hydrolysis [8].

Hydrogenase and ferredoxin

These were prepared as indicated in the following references: *Clostridium pasteurianum* hydrogenase [9], but modified as previously described [10]; *Rhodospirillum rubrum* hydrogenase, [11]; *Escherichia coli* hydrogenase, [12]; *Spirulina maxima* ferredoxin, [13].

H_2-evolution assay

The 2 ml reaction mixture, containing the hydrogenase and sodium dithionite (10 mM) in 50 mM phosphate buffer, pH 7.0, was incubated under N_2 at 30°C in 15 ml sealed vials in a shaking water-bath. The clusters were added by syringe to start the reaction. Where Me_2SO was used as solvent for the Fe-Mo dimer and the Fe_4Se_4 cluster, the maximal final concentrations were 1.5% and 10% (v/v) respectively. H_2 was determined as described previously [10]. All

results are the average values from three separate experiments using two different preparations of the same cluster; for the Fe_4Se_4 cluster one preparation only was used. One unit of hydrogenase activity catalysed the evolution of 1 μmol H_2 per h with methyl viologen (2.5 mM) as the electron carrier.

H_2-evolution from the chloroplast-hydrogenase system

This was performed as described previously [14]. In 15 ml vials, the 2 ml reaction mixture contained spinach chloroplasts equivalent to 100 μg chlorophyll, 100 nmol glucose, 20 units of glucose oxidase, 170 nmol ethanol, 1000 units of catalase, 10 mg bovine serum albumin (fat-free, fraction V) in 20 mM potassium phosphate buffer, pH 7.0, under a N_2 atmosphere at 25°C in a shaking water-bath. The system was illuminated at a light intensity of 11 000 lux. Ferredoxin or the cluster (see text) were then injected and the reaction was started by the addition of *C. pasteurianum* hydrogenase (30 units). H_2 was determined as above.

Results and Discussion

The synthetic model clusters $[Fe_6Mo_2S_8(S \cdot CH_2CH_2OH)_9]^{3-}$, $[Fe_4S_4(S \cdot CH_2CH_2OH)_4]^{2-}$ and $[Fe_4Se_4(S \cdot CH_2CH_2OH)_4]^{2-}$ (henceforth termed the FeMo dimer, the Fe_4S_4 cluster and the Fe_4Se_4 cluster, respectively) were tested to see if they would replace the various components of a H_2-evolving system.

FeMo dimer

The FeMo dimer was used as solution in Me_2SO (Me_2SO dimer) or in Tris-HCl buffer, pH 8.5, with added mercaptoethanol (Tris dimer, see Methods). As shown in Table I, in the absence of hydrogenase the FeMo-dimer was unable to catalyse H_2 evolution from dithionite as the electron source, with either ferredoxin or methyl viologen as the electron carrier. Thus the FeMo dimer would

TABLE I

H_2 EVOLUTION WITH THE FeMo-DIMER

The 2 ml reaction mixture (see text) contained the hydrogenase and electron carrier as indicated. The abbreviations and concentrations used were: Cps, *C. pasteurianum* (17.6 units); Ec, *E. coli* (10.5 units); Rr, *R. rubrum* (9.0 units); Fd, *S. maxima* ferredoxin (50 μM); MV, methyl viologen (2.5 mM). The concentrations of the Tris dimer and Me_2SO dimer were 2.5 mM and 0.4 mM respectively.

Hydrogenase	Electron carrier	H_2 evolved after 10 min (μmol)
Cps, Ec or Rr	None	0
Cps	Fd	1.73
Ec or Rr	Fd	0
Cps	MV	2.93
Ec	MV	1.75
Rr	MV	1.51
Cps	Tris dimer	2.86
Cps	Me_2SO dimer	0.225
Ec	Tris or Me_2SO dimer	0
Rr	Tris or Me_2SO dimer	0
Tris or Me_2SO dimer	MV	0
Tris or Me_2SO dimer	Fd	0

not function as a hydrogenase: Table I also shows the ability of the FeMo dimer to act as an electron carrier to hydrogenase from dithionite. *C. pasteurianum* hydrogenase readily evolved H_2 from the reduced Me_2SO dimer or Tris dimer but the hydrogenases of *E. coli* and *R. rubrum* would not. It may be noted that the hydrogenases of *E. coli* and *R. rubrum* are unable to use ferredoxin as an electron carrier [11,12] but readily evolve H_2 from dithionite-reduced methyl viologen. The FeMo dimer would thus replace ferredoxin in the H_2 evolving system but is not a general mediator since it would not substitute for methyl viologen. Since *C. pasteurianum* hydrogenase was obligatory for H_2 production in a system containing dithionite and the FeMo dimer, the H_2 evolved does not originate directly from the breakdown of the FeMo dimer but arises from a hydrogenase-catalysed redox reaction.

The effect of the dimer concentration on the rate of H_2 evolution is shown in Fig. 1. The Tris dimer was more active than the Me_2SO dimer but the kinetics were the same. Both showed increasing activity with increasing concentration but between 70—200 μM, the kinetics were very unusual. At dimer concentrations above 400 μM, the Tris dimer was used instead of the Me_2SO dimer to eliminate any effects of Me_2SO on the enzyme activity (see Ref. 15). Fig. 2 shows that the H_2 evolution activity increases with increasing FeMo-dimer concentration reaching a maximum at about 2.0 mM, when the activity was similar to that obtained with saturating concentrations of ferredoxin (50 μM). The

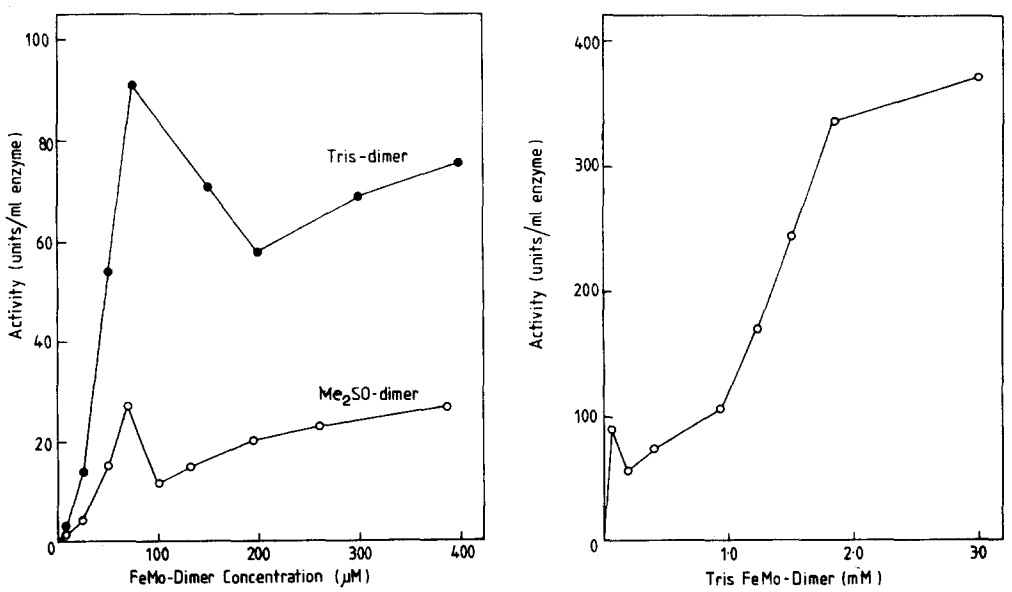

Fig. 1. H_2 evolution with the FeMo-dimer as the electron carrier. The 2 ml reaction mixture (see text) contained *C. pasteurianum* hydrogenase (50 μl, 350 units/ml) with the Tris-dimer (•) or the Me_2SO-dimer (○) as the electron carrier. The activity was calculated from the first 10 min of H_2 evolution.

Fig. 2. Effect of Tris FeMo-dimer concentration on the rate of H_2 evolution. The 2 ml reaction mixture (see text) contained *C. pasteurianum* hydrogenase (50 μl, 350 units/ml) with the Tris-dimer as the electron carrier. The activity was calculated from the first 10 min of H_2 evolution.

apparent K_m of the Tris dimer for hydrogenase (calculated as the substrate concentration giving half-maximal velocity, approx. 1.2 mM) is greater than the apparent K_m value of 51 μM reported for *C. pasteurianum* ferredoxin but lower than that of methyl viologen (6.25 mM, [9]).

The rate of H_2 production with the Tris dimer as the electron carrier remained constant for less than 5 min before rapidly decreasing; only trace amounts of H_2 were evolved after 30 min. In contrast, the rate of H_2 evolution with the Me_2SO dimer was constant for well over 30 min. Thus, the Tris dimer was more active but less stable than the Me_2SO dimer. However, both dimers were much less stable than methyl viologen or ferredoxin, which support H_2 evolution at a constant rate for several hours. The mercaptoethanol added to the Tris dimer was not responsible for the increased activity as the activity of the Me_2SO dimer in the H_2 evolution assay was not stimulated by the presence of mercaptoethanol.

The decreased rate of H_2 production with the FeMo dimer as the electron carrier was assumed to arise from the instability of the dimer in aqueous solution. However, if fresh Me_2SO dimer, dithionite or hydrogenase were added, alone or in combination, to a reaction mixture containing the Me_2SO dimer, dithionite and hydrogenase after a 1-h incubation (when H_2 production had stopped), there was no further H_2 evolution. Also addition of methyl viologen after 1 h did not result in further H_2 evolution. Similar results were obtained with the Tris dimer. Thus, it would appear that inhibition of the hydrogenase by material arising from the decomposition of the FeMo dimer also contributes to the observed decrease in the rate of H_2 production.

Fe_4S_4 cluster

The Fe_4S_4 cluster was unable to evolve H_2 with either methyl viologen or

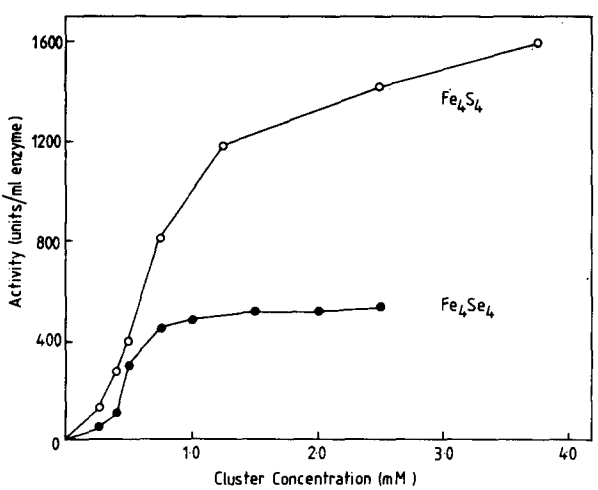

Fig. 3. Effect of Fe_4S_4- or Fe_4Se_4-cluster concentration on the rate of H_2 evolution. The 2 ml reaction mixture (see text) contained *C. pasteurianum* hydrogenase (50 μl, 404 units/ml) with the Fe_4S_4- (○) or the Fe_4Se_4-cluster (●) as the electron carrier. The activity was calculated from the first 10 min of H_2 evolution.

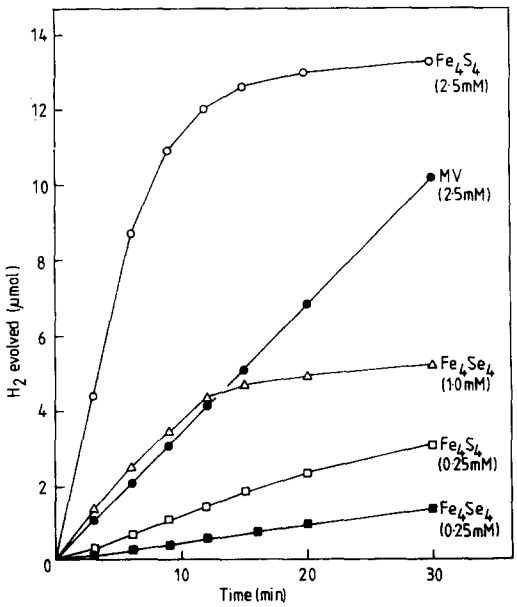

Fig. 4. Duration of H_2 evolution with the Fe_4S_4- or Fe_4Se_4-cluster as the electron carrier. The 2 ml reaction mixture (see text) contained *C. pasteurianum* hydrogenase (50 μl) with either methyl viologen (MV), the Fe_4S_4-cluster (Fe_4S_4) or the Fe_4Se_4-cluster (Fe_4Se_4) as the electron carrier at the indicated concentration.

ferredoxin as the electron carrier from dithionite and thus would not function as an hydrogenase. However, the Fe_4S_4 cluster in Tris solution would replace ferredoxin in the H_2-evolving system and mediate electron transfer to *C. pasteurianum* hydrogenase from dithionite, as with the same cluster used as a Me_2SO solution [6]. As shown in Fig. 3, saturation was observed at a cluster concentration of 3—4 mM. The initial rate of H_2 production was about 4-fold greater than that obtained with either methyl viologen or ferredoxin as the electron carrier (Fig. 4). The rate of H_2 evolution remained constant for less than 10 min though at lower concentrations of the cluster, H_2 production continued for a longer period (Fig. 4). Unusual kinetic behaviour was observed when low Fe_4S_4 cluster concentrations were used (Fig. 5). By comparison with previous results [6], the Fe_4S_4 cluster was more active in Me_2SO than in Tris solution (up to a final concentration of 0.3 mM) although the kinetics were similar. As with the FeMo dimer, the Fe_4S_4 cluster supported H_2-evolution for longer periods in Me_2SO solution than when used in Tris solution. The Fe_4S_4 cluster was also unable to mediate electron transfer to *E. coli* or *R. rubrum* hydrogenases.

Fe_4Se_4 cluster

The Fe_4Se_4 cluster was used as a solution in Me_2SO. The cluster would not function as a hydrogenase, nor would it act as an electron carrier to *R. rubrum* or *E. coli* hydrogenases with dithionite as the electron donor. It would however, replace ferredoxin in a H_2-evolving system with *C. pasteurianum* hydro-

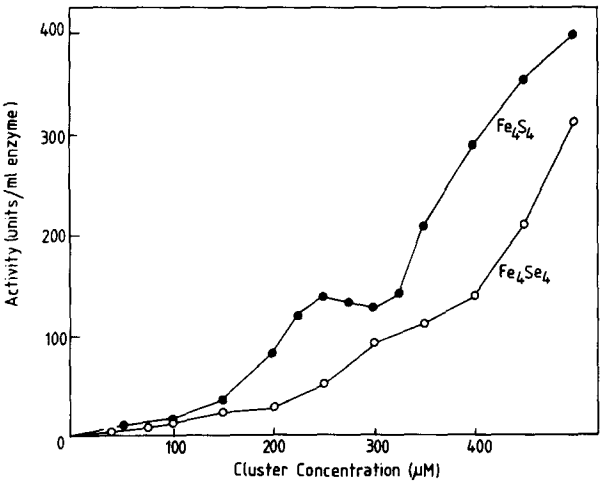

Fig. 5. Effect of cluster concentration on the rate of H_2 evolution. The 2 ml reaction mixture (see text) contained *C. pasteurianum* hydrogenase (50 μl, 404 units/ml) with the Fe_4S_4-cluster (•) or the Fe_4Se_4-cluster (○) as the electron carrier. The activity was calculated from the first 10 min of H_2 evolution.

genase and dithionite. The results, given in Figs. 3 and 4, show that the rates of H_2 evolution obtained with the Fe_4Se_4 cluster were slightly lower than those obtained with the Fe_4S_4 cluster and the hydrogenase was saturated at a lower concentration (about 1 mM Fe_4Se_4). It should be noted that relatively high Me_2SO concentrations were used (up to 10%, v/v). Again, low concentrations of the Fe_4Se_4 cluster supported H_2 evolution for a longer period. Fig. 5 shows that with the Fe_4Se_4 cluster, the unusual kinetic behaviour seen with the other clusters described above was not apparent.

In conclusion, all the clusters tested would replace ferredoxin in the dithionite H_2-evolving system but will not function as general bipyridyl-type redox carriers nor as hydrogenases. All three clusters will support high rates of H_2 evolution with *C. pasteurianum* hydrogenase, at least comparable to those obtained with ferredoxin or methyl viologen as the electron carrier. In view of the fact that when H_2 production ceases, the hydrogenase was inactivated, the observed decrease in the duration of H_2 evolution seems to arise in part from inhibition of the hydrogenase by breakdown products of the cluster.

Photoactivated H_2 evolving systems

Methyl viologen may be photoreduced by irradiation with visible light in the presence of proflavine with EDTA as the electron donor. If hydrogenase is also present at time zero, H_2 is evolved upon illumination of the system (see Refs. 16—18). Ferredoxin will substitute for methyl viologen in this system and will couple to *C. pasteurianum* hydrogenase with the evolution of H_2. As yet we have not been able to show that the FeMo dimer, the Fe_4S_4 cluster or the Fe_4Se_4 cluster can replace ferredoxin in this activated H_2-evolving system.

Ferredoxins may be reduced by illuminated chloroplasts which in the presence of hydrogenase leads to the evolution of H_2 [10,19]. Attempts to substitute ferredoxin with the Fe_4S_4 cluster or the Fe_4Se_4 cluster were unsuc-

TABLE II

REPLACEMENT OF FERREDOXIN BY THE FeMo-DIMER IN THE CHLOROPLAST-HYDROGENASE SYSTEM

The 2 ml reaction mixture (see text) contained *S. maxima* ferredoxin or the Tris FeMo-dimer as the electron carrier at the concentration indicated.

Electron carrier	Concentration (μM)	H_2 evolved in 1 h (μmol H_2/mg chlorophyll)
None	—	0
Ferredoxin	50	28.0
FeMo-dimer	50	0.7
FeMo-dimer	250	2.0
FeMo-dimer	500	8.3
FeMo-dimer	1000	11.2

cessful, there was no H_2 evolution. However, the FeMo dimer in Tris solution (but not in Me_2SO solution) would replace ferredoxin. As shown in Table II, the rate of H_2 production catalysed by *C. pasteurianum* hydrogenase increased with increasing FeMo dimer concentrations up to 1 mM. The optimal ferredoxin concentration in this system is about 50 μM [14]. We feel that the ability of the FeMo dimer to substitute for ferredoxin is an important step towards replacing the biological electron transfer components and the chloroplast-hydrogenase system with synthetic analogues.

Acknowledgements

This work was supported by the U.K. Science Research Council and the European Commission. We thank Dr. R. Cammack for advice and discussion and Mr. P. Gisby for the preparation of the chloroplasts.

References

1 Cramer, S.P., Hodgson, K.O., Gillum, W.O. and Mortenson, L.E. (1978) J. Am. Chem. Soc. 100, 3398—3407
2 Cramer, S.P., Gillum, W.O., Hodgson, K.O., Mortenson, L.E., Stiefel, E.I., Chisnell, J.R., Brill, W.J. and Shah, V.K. (1978) J. Am. Chem. Soc. 100, 3814—3819
3 Christou, G., Garner, C.D., Mabbs, F.E. and King, T.J. (1978) J. Chem. Soc. Chem. Commun., 740—741
4 Christou, G., Garner, C.D., Mabbs, F.E. and Drew, M.G.B. (1979) J. Chem. Soc. Chem. Commun., 91—92
5 Christou, G., Garner, C.D. and Mabbs, F.E. (1978) Inorg. Chim. Acta 28, L189—190
6 Adams, M.W.W., Reeves, S.G., Hall, D.O., Christou, G., Ridge, B. and Rydon, H.N. (1977) Biochem. Biophys. Res. Commun. 79, 1184—1191
7 Christou, G. and Garner, C.D. (1979) J. Chem. Soc. Dalton, in the press
8 Hill, C.L., Renaud, J., Holm, R.H. and Mortenson, L.E. (1977) J. Am. Chem. Soc. 99, 2549—2556
9 Chen, J.S. and Mortenson, L.E. (1974) Biochim. Biophys. Acta 371, 283—298
10 Rao, K.K., Rosa, L. and Hall, D.O. (1976) Biochem. Biophys. Res. Commun. 68, 21—28
11 Adams, M.W.W. and Hall, D.O. (1979) Arch. Biochem. Biophys. 195, 288—299
12 Adams, M.W.W. and Hall, D.O. (1978) Biochem. Soc. Trans. 6, 1339—1341
13 Hall, D.O., Rao, K.K. and Cammack, R. (1972) Biochem. Biophys. Res. Commun. 47, 798—802
14 Rao, K.K., Gogotov, I.N. and Hall, D.O. (1978) Biochemie 60, 291—296
15 Reeves, S.G. and Hall, D.O. (1977) Cell Biol. Intl. Reports 1, 353—361
16 Chan, M.S., Evans, C.A., Markiewicz, S., Sparks, R. and Bolton, J.R. (1976) in Book of Abstracts.

First International Conference on Photochemistry. Conversion and Storage of Solar Energy, PE7, London, Ontario
17 Krasna, A.I. (1979) Photochem. Photobiol. 29, 267—276
18 Adams, M.W.W., Rao, K.K. and Hall, D.O. (1979) Photobiochem. Photobiophys., in the press
19 Benemann, J.R., Berenson, J.A., Kaplan, N.O. and Kamen, M.D. (1973) Proc. Natl. Acad. Sci. U.S.A. 70, 2317—2320