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THE ACCEPTOR SPECIFICITY OF FLAVINS AND FLAVOPROTEINS

III. FLAVOPROTEINS

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SUMMARY

1. The specificity of flavoproteins towards acceptors has been rather neglected, but an attempt is here made to construct a comparative table of acceptor specificities of those flavoprotein enzymes for which data exist.

2. The acceptor specificity of reduced flavin groups, when combined with apoenzyme proteins, is quite different from that of the same flavin groups in the free state (see Part II). Free flavins react very rapidly with a wide range of acceptors, but the same groups combined as flavoproteins have a severely restricted range of action.

3. There are remarkable differences between different flavoproteins. Nearly every flavoprotein fails altogether to react with at least one, and often several, of the acceptors, giving a specificity pattern which is different in each case. There seems to be no general acceptor for flavoproteins.

4. The effect of combination of a flavin with a particular apoenzyme is to inhibit specifically the reaction of the flavin with particular acceptors with which it would react very rapidly in the absence of the apoenzyme.

5. Each apoenzyme produces its own distinctive pattern of inhibitions. The degree of inhibition is often very high; the table shows over 50 cases of specific inhibitions that are essentially complete. Some of these are very difficult to explain.

6. There is no obvious parallelism between any acceptor and any other in its pattern of reactivity with a series of different flavoproteins.

7. In a few cases combination with apoenzyme specifically accelerates the reaction of the flavin with particular acceptors, so that the flavoprotein is oxidized faster than the free flavin.

8. Possible correlations are discussed between the effects of apoenzymes on the reactivity of flavins with acceptors and a number of special known features of different apoenzymes, but no adequate explanation of the differences in specificity has emerged.

9. In view of the interesting nature of the effects, a plea is made for a more intensive study of the acceptor side of flavoprotein specificity.

Abbreviations: DCIP, dichlorophenolindophenol; PMS, phenazine methosulphate.

INTRODUCTION

In the preceding paper[1] it is shown that free reduced flavins react very rapidly with a wide variety of acceptors; their behaviour in fact is very similar to that of dithionite, an unspecific chemical reducing agent. There is no obvious reason why combination of the flavins with proteins (apoenzymes) should prevent this, and it might have been expected that the flavoprotein enzymes would also react rapidly with a very large number of acceptors. The actual situation is quite otherwise. Combination of the flavin with an apoenzyme has a profound effect on its reactivity towards acceptors. It may abolish the power of reacting with certain acceptors, while leaving the reaction with others unaffected. In a few cases it may, on the other hand, confer acceptor properties on substances which do not react with free flavins.

The different apoenzyme proteins differ strikingly from one another in their effects on the flavin combined with them. For example, combination with one protein will inhibit the reaction of the reduced flavin with acceptors B, C, E, F and H, while combination with another will inhibit the reaction of the same flavin with acceptors A, C, D, F and G. The available data suggest that each of the proteins produces its own specific pattern of acceptor inhibitions. Moreover the effects of different proteins on the reactivity of one acceptor do not seem to run parallel with their effects on the reactivity of another. That is to say the reaction of the flavin with acceptor A may be inhibited by apoenzymes 1, 3, 4, 7 and 8, but with acceptor B by apoenzymes 2, 3, 5 and 8. There appears to be at present no known acceptor that will react with all flavoproteins.

The object of the present paper is not so much to provide an explanation of these effects as to draw attention to their existence and interesting nature, and to make a plea that, as purified flavoproteins become available for examination, a more detailed study of their acceptor specificity should be made.

FLAVOPROTEIN ACCEPTOR SPECIFICITY

Of the fifty or more known flavoprotein enzymes, only a minority have been studied with a range of acceptors; more often only two or three acceptors have been used. Thus the results in the literature are very fragmentary, except in about twenty cases. In general, pure flavoproteins are not on the market, and the isolation of a flavoprotein in a sufficiently purified state for specificity studies is a major undertaking. It was therefore hardly practicable in the present work to carry out anything like a full study of flavoprotein acceptor specificity, although a number of gaps in the published data have been filled by original observations. This discussion is consequently restricted in the main to those enzymes that have been tested with a range of acceptors.

Table I summarizes the behaviour of twenty flavoprotein enzymes towards the series of acceptors tested with free flavins and shown in Table II of the preceding paper[1]; the data are collected from the literature, supplemented by original observations. The tests on the different enzymes have been done under such a wide variety of conditions, and the results expressed in so many different ways, that it is hardly possible to give them in comparative numerical terms, and it was considered preferable to express them semi-quantitatively in terms of number of *plus* signs,

which in fact shows them more clearly. For any one enzyme, the relative rates obtained with the different acceptors are probably shown fairly correctly. Comparison of the activities of different enzymes, however, are less reliable. This is because in many cases it has been the practice in the literature to give the rates with different acceptors only on a relative basis, taking the rate with the best accepter as 100. In other cases the molar concentration of the flavoprotein was unknown, so that the rates could not be given in absolute terms. Moreover, different enzymes were often tested under different conditions, *e.g.* of pH and temperature, which of itself makes comparison difficult.

Nevertheless, molecular activities ('turnover numbers') have been determined in a fair proportion of cases, and in assembling the table an attempt has been made to give a reasonably correct picture. A zero denotes that no reaction was detectable, while $\pm$ denotes that a very slow reaction could be definitely detected.

It must be emphasized that the data of Table I are based mainly on rates of the overall catalytic reactions, not on the rates of oxidation of the fully reduced forms of the flavoproteins by the acceptors, which are known only in very few cases. No doubt the overall rate is often partly limited by the earlier steps in the enzyme reaction, that is to say by the steps involved in the reduction of the flavin group by the substrate. It must be presumed, however, that when an enzyme works rapidly with one acceptor the flavin group is readily reduced by the substrate, and if there is no action with another acceptor it is because the reduced flavin group is not being oxidized by this acceptor. The zeros in the table are thus highly significant, while a difference between $+++$ and $++$ is less so.

It is not implied that the reduced form of the flavin group which reacts with the acceptor is the same in all cases. In some cases it is the fully reduced form, in others a semiquinone or some other form, and in the majority its nature is uncertain. It is highly desirable that an extensive study by the most modern methods should be made of the reaction rates of known reduction states of flavoproteins with different acceptors.

Although Table I includes less than half of the known flavoproteins, several things are immediately obvious. The behaviour of flavin nucleotides combined with these proteins is very different from that of the same flavins in the free state. Apart from nitrate and hydrogen peroxide, all these acceptors react very rapidly with free flavins, but this is very far from being the case with the flavoproteins. There is not one that reacts significantly with all the acceptors. Conversely there appears to be no general acceptor for flavoproteins; of even the best acceptors, each one fails to react with at least three of the different flavoproteins shown, and some with many more than this. Apparently no two flavoproteins have identical acceptor specificity patterns. Each of the proteins impresses its own characteristic pattern of reactivity of the flavin when it is combined with it.

This pattern is the result of a strong inhibiting action of the protein on the reaction between the flavin combined with it and certain particular acceptors, different in each case, while the reaction with other acceptors is unaffected. Each of the fifty or more zeros in Table I (except those in the nitrate column) represents a specific inhibition by the protein of a reaction that would occur very rapidly in its absence. There is no obvious parallelism between one acceptor and another with respect to the group of enzymes with which they react, and it is not possible to predict the behaviour

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TABLE I

ACCEPTOR SPECIFICITY OF SOME FLAVOPROTEINS

Abbreviations: B, bacteria; K, kidney; LM, liver microsomes; SV, snake venom; Y, yeast; DCIP, dichlorophenolindophenol; PMS, phenazine methosulphate.

EC number	*Flavoprotein*	*Oxygen*	H_2O_2	*Ferricyanide*	*Benzoquinone*	*Cyt. c*	*DCIP*	*Methylene blue*	*PMS*	*Nitrate*	*Ref.*
1.1.3.4	Glucose oxidase	++++		±	o	o	+	+	+		2
1.1.99.8	Glucose dehydrogenase	o			++		+++	+			3
1.1.3.1	Glycollate oxidase	++		o		o	+	+		o	4
1.1.3.2	L-Lactate oxidase (or oxygenase) (B)	++		o		o	+	o			5, 6
1.1.2.3	L-Lactate dehydrogenase (Y)	±		+++		+++	+++	+++			7, 8
1.1.2.4	D-Lactate dehydrogenase (Y)	o		o		++	o	o	+++		9
1.1.99.6	D-2-Hydroxyacid dehydrogenase	+		++		+	++	++	+++		10
1.4.3.2	L-Amino-acid oxidase (SV)	+++		±	o	o	±	+	+++		11
1.4.3.3	D-Amino-acid oxidase (K)	+++		o	o	o	+	+	o		12
1.4.3.1	D-Aspartate oxidase (K)	++		+++	o	o	+	o	o		13
1.2.3.2	Xanthine oxidase	++	+	±	++	o	+++	+++	+++	++	14, 15
1.3.3.1	Dihydro-orotate dehydrogenase	+++		+			+	+	+		16
1.5.99.1	Nicotine dehydrogenase	o	o	o	+	o	+	+++			17
1.6.5.1	Quinone reductase (B)	o	o	++	++++	o	+++	o		o	18
1.11.1.1	NAD peroxidase	±	+++	+++	+	o	o	o		o	19
1.6.99.5	NAD(P)H dehydrogenase	o		+++	o	o	++	o			20
1.6.2.3	NADPH–cytochrome *c* reductase (LM)	o		+++	+++	+++	+++				21, 22
1.6.2.2	NADH–cytochrome b_5 reductase (LM)	o		+++	±	o	+	+			23, 24
1.3.99.1	Succinate dehydrogenase	o		++	o	o	o	o	+++		25, 26
1.6.4.3	Lipoamide dehydrogenase	±		++	o	o	++	++	o		27, 28

of an enzyme towards a given acceptor from its reactivity with other acceptors. This is a remarkable situation, and one that is very difficult to explain.

The nature of the effects will be seen more clearly if the acceptors are considered individually.

INDIVIDUAL ACCEPTORS

Oxygen

The fact that reduced flavins, when uncombined with proteins, are so rapidly oxidized by O_2 would lead one to expect that flavoproteins in general would be similarly oxidized. This is far from being the case; of the enzymes in Table I, more than half have little or no activity with O_2 as acceptor. This would be less surprising if the reduced flavin were combined with the protein in such a way as to make it non-oxidizable; but, on the contrary, in every case where O_2 fails to oxidize the flavin, one or more of the other acceptors will bring about the oxidation very rapidly.

The inhibition of the reaction with O_2 by many of the apoenzymes is indeed difficult to explain. It can hardly be a question of accessibility; one can scarcely imagine that the flavin group could be buried within the protein molecule in such a way as to make it inaccessible to such a small uncharged molecule as O_2 while leaving it accessible to much larger acceptor molecules, even in some cases to such relatively enormous molecules as cytochromes.

Neither can it be due to a change of the redox potential of the flavin on combination with the protein, for O_2 lies well above the other acceptors on the redox scale, and would be the last to be affected by a change of potential. Among the different flavoproteins there does not appear to be any correlation, either positive or negative, between reaction with O_2 and reaction with any of the other acceptors.

It has been questioned whether the rate of reaction of any of the reduced flavoproteins with O_2 is absolutely zero. In one or two cases, previously thought not to react, a slow reaction was detected when very large concentrations of the enzyme were used. But this does not really affect the argument. There is at any rate an enormous reduction in the reactivity of the flavin towards O_2 when it combines with many proteins, although other proteins have no such effect. For example in quinone reductase the rate of oxidation of the flavin by O_2 is reduced to 1/100000 of its rate of oxidation by quinone, and in cytochrome b_5 reductase the flavin is oxidized by cytochrome b_5 20000 times as rapidly as by O_2. In the latter case the time of oxidation by O_2 is changed from a few milliseconds for the free flavin to over a minute for the flavoprotein, an inhibition of the order of 99.9 % by the protein.

Of those flavoproteins that do react with O_2, most are oxidized at about the same rate as free flavin or rather more slowly, but one at least, glucose oxidase, is oxidized by O_2 even faster than the free flavin, suggesting that here there is a definite catalysis of the oxidation by the protein.

As mentioned in the preceding paper[1], there is some evidence that the reaction of O_2 with reduced flavins in the free state may take place in more than one way, and the exact nature of the reaction of flavoproteins with O_2 is not entirely certain. The conversion of molecular O_2 to H_2O_2, which requires 2 reducing equivalents, is brought about by a great variety of reducing systems, including leuco-dyes, dithionite, and certain enzymes containing metal atoms but no flavin, as well as by

reduced flavins. It is generally assumed that it occurs by a simple 2-equivalent reduction of the O_2. Recently, however, McCORD AND FRIDOVICH[29] have shown that in the case of xanthine oxidase the O_2 is converted by a 1-equivalent reduction into the free radical of peroxide, namely $\cdot O_2H$ or its anion $\cdot O_2^-$ (superoxide anion) which very rapidly dismutes into O_2 and H_2O_2 in aqueous solution.

This may be so with some other flavoprotein oxidases also, but it is not safe to argue from xanthine oxidase, which is an exceptionally complicated enzyme, containing 1 atom of molybdenum and 4 atoms of iron for each flavin group[30]. The molybdenum is known to be concerned with the reduction of the flavin by the substrate[31], but it is still not certain whether it is the flavin or the iron that reacts with the O_2[32]. It is true that KOMAI *et al.*[33] were able to remove the flavin reversibly from the enzyme, and showed that the de-flavo-enzyme no longer reacts with O_2, but catalyses the oxidation of the substrate by ferricyanide, a reaction that is very slow when the flavin is in position. This, however, is not necessarily inconsistent[32] with the reaction scheme of BRAY *et al.*[34], namely S→Mo→FAD→Fe→O_2, in which the O_2 reacts with iron rather than with flavin. RAJAGOPALAN AND HANDLER[35] state that 'among a multitude of enzymes tested, only aldehyde oxidase and dihydro-orotate dehydrogenase were found to mimic xanthine oxidase in eliciting the O_2 radical dependent reactions'; these three flavoproteins all contain iron. They also speak of 'The inability of simple flavoproteins to generate O_2 radicals'.

This view, however, has very recently been disputed by MASSEY *et al.*[51], who have shown, using the inhibition of reduction of cytochrome *c* aerobically by erythrocuprein (superoxide dismutase) as a test for $\cdot O_2^-$, that several metal-free flavoproteins do form this radical at pH 8.5. Moreover intact xanthine oxidase forms the radical, but after removal of the flavin group it does not. It has also been shown[52] that a number of iron- (but not flavin-) containing proteins do not form the radical.

The ability to react with O_2 must be very sensitive to small structural changes, for purified xanthine oxidase from chicken or pigeon liver differs from the mammalian enzyme in being unable to react with O_2, although it has the same physical properties and content of flavin, molybdenum and iron[35].

There is as yet no adequate explanation for the specificity of flavoproteins towards O_2, but while this paper was being written MASSEY *et al.*[36] published the first promising clue, namely that the flavin groups of those enzymes that react with O_2 resemble the free flavins in combining with sulphite, while those that do not react with O_2 do not form sulphite complexes. Moreover there is a correlation (though with a few exceptions) between the spectral type of the semiquinone form of the flavoprotein and reactivity with O_2 and sulphite. This reactivity tends to be shown by the enzymes that give a red (or anionic) semiquinone, while the non-reactive ones give a blue (or neutral) semiquinone. It was suggested that the presence of a positively charged group in the protein near the flavin might account for both the sulphite binding and the semiquinone type. It is not clear, however, why it should influence the reactivity with O_2; the sulphite combines with the oxidized, but not the reduced, form of the flavin[37], whereas it is the reduced form that racts with the O_2.

Ferricyanide

At one time this was regarded as a rather general acceptor for flavoproteins, but in fact nearly half those tested fail to reduce it. This is not due to an inactivation

of the enzyme by the ferricyanide in any of the cases that I have tested. Since the free flavins react very rapidly with ferricyanide, it must be due to inhibition by the proteins.

There is no apparent correlation between reactivity with ferricyanide and any other acceptor, with the doubtful exception of a negative correlation with O_2. It is true that there are many cases that react with O_2 but not ferricyanide, or with ferricyanide but not O_2; but there are also cases that react well with both (*e.g.* D-aspartate oxidase) or with neither (*e.g.* D-lactate dehydrogenase of yeast).

A striking case of specificity towards ferricyanide is given by the D-amino acid and D-aspartate oxidases of kidney; for although they both act on D-amino acids and catalyse the same type of reaction, the former does not use ferricyanide at all[12], while the latter reacts with it better than with any other acceptor[13]. This difference may be connected with the most distinctive property of ferricyanide, namely its high negative charge. D-Amino-acid oxidase oxidizes most amino acids, but not the dicarboxylic ones aspartic and glutamic acids; on the other hand D-aspartate oxidase oxidizes these two dicarboxylic amino acids, but has no action on other amino acids. This suggests that the protein of the aspartate oxidase carries a positive charge in the neighbourhood of the substrate-binding site, which would attract the negatively charged substrates and ferricyanide, while the D-amino-acid oxidase carries also a negative charge, which would repel these two substrates and ferricyanide. The difference between the two oxidases is seen also in the fact that ferricyanide strongly inhibits the reaction of D-aspartate oxidase with O_2, but has no effect on the O_2 uptake of D-amino acid oxidase.

The presence of a negatively charged group in the neighbourhood of the binding site is in no way inconsistent with the presence of the well-authenticated positive group which is believed to combine with the carboxyl group of the substrates. The above interpretation is essentially that put forward by MASSEY AND GANTHER[53] to explain the lack of binding of dicarboxylic acids by D-amino-acid oxidase, who say that a carboxyl group as the primary requirement for ability to bind to the active centre 'implies the existence in the enzyme of a positively charged group . . . presumably chose to the . . . flavin. The lack of binding of dicarboxylic acids . . . implies also the existence of a negatively charged group in the vicinity of the active centre'.

Another interesting case is that of NADH–cytochrome *c* reductase (EC 1.6.2.1) (NADH dehydrogenase) of mitochondria[38]. As first extracted in a particulate form as 'Complex I' of the respiratory system, the enzyme is 'protected by a hydrophobic sheath of lipids and structural proteins' and has a very low activity with acceptors other than ferricyanide. It can, however, be freed from the sheath by several mild procedures, with a great reduction in molecular weight, and obtained as a soluble flavoprotein. It then reacts readily with ferricyanide, quinones, cytochrome *c* and DCIP as acceptors; but whereas the rate with ferricyanide is not increased (indeed it is somewhat diminished) by the treatment, that of the other acceptors is increased 10-fold or more. Thus before the sheath is removed ferricyanide is the only effective acceptor, but after its removal all the acceptors react at roughly the same rate. What is surprising is that the sheath does not prevent the access of ferricyanide to the enzyme; but it is not impossible that the ferricyanide might itself produce a change in

the colloidal state of the particle, for it is well known that ions with multiple charges may profoundly influence the state of colloidal systems.

It cannot be assumed that when ferricyanide acts as an acceptor with flavoproteins it always reacts with the flavin group, for as already mentioned it reacts with xanthine oxidase after the flavin group has been removed[33], and in that case it presumably reacts either with the molybdenum or with the iron.

Quinones

It was pointed out in the preceding paper[1] that benzoquinone is one of the most reactive acceptors with free flavins, and it is all the more surprising that with flavoproteins it more often than not fails to react at all or reacts only slowly. This again is difficult to explain, as it is not a particularly large molecule and carries no charge.

On the other hand, in one case (quinone reductase) it reacts so rapidly as to suggest that there is a specific activation of the quinone by the enzyme. As would be expected if that were the case, there is some degree of specificity within the quinone series. With benzoquinone as acceptor, the enzyme undergoes about 1500 cycles of reduction and oxidation per sec, but only 10 cycles/sec with menadione (2-methyl-1,4-naphthoquinone)[18], while menadione reductase (EC 1.6.5.2) undergoes about 10000 cycles/sec under favourable conditions with menadione as acceptor, but less than one-third of this with benzoquinone[39]. (For comparison, 50 cycles/sec might be taken as a fairly usual activity with other flavoproteins, although there are wide variations.)

Cytochromes

The distinctive feature of the cytochromes is their high molecular weight compared with other acceptors. This would be expected to accentuate any inhibitory effect of the enzyme proteins on reaction with the flavin group by hindering the access of the haem to the flavin. In fact, although cytochrome *c* rapidly oxidizes free reduced flavins, it reacts with fewer flavoproteins than any of the other acceptors, with the possible exception of nitrate and hydrogen peroxide, for which only meagre data are available.

One or two flavoproteins, for example xanthine oxidase, which had been thought to reduce cytochrome *c* do not in fact do so unless O_2 is present[40]. McCORD AND FRIDOVICH[32,41] showed that here the cytochrome was not in fact reacting with the flavoprotein, but was reduced non-enzymically by the peroxide radical $\cdot O_2^-$ produced by the reduction of O_2 by oxidases of this type.

When acting as acceptors, the cytochromes seem to be rather specific as between themselves. For example cytochrome b_5 reductase is $+++$ with cytochrome b_5 but 0 with cytochrome *c*, while cytochrome c_2 reductase (EC 1.6.2.5) is $+++$ with cytochrome c_2 but only $\pm$ with cytochrome *c*[42].

DCIP, methylene blue, phenazine methosulphate (PMS)

The readily reducible dye DCIP comes nearest to being a general acceptor for flavoproteins. Nevertheless there are a few that do not react with it, and quite a number that only react slowly.

In some cases at least the reactivity towards DCIP may vary greatly with the condition of the enzyme. VEEGER AND MASSEY[43] showed that when lipoamide

dehydrogenase is freshly prepared it reacts with DCIP only one-tenth as fast as it does with oxidized lipoate. But if it is incubated in the presence of small traces of copper salts the rate with DCIP gradually increases and that with oxidized lipoate decreases, until after ten hours of incubation there has been a 20-fold increase in the DCIP rate and a decrease in the rate with lipoate to one-tenth of the initial value. This change they attribute to catalysis by the copper of the oxidation of essential –SH groups in the enzyme protein. Presumably these groups are necessary for the oxidation of the reduced flavin by oxidized lipoate, but inhibitory for its oxidation by DCIP.

Methylene blue, the classical acceptor for dehydrogenases, shows a tendency to run parallel with DCIP, although the parallelism is by no means perfect. Of the cases given in the table, 12 give the same results with both dyes, 6 are definitely different, and in 5 of these 6 cases DCIP is the better acceptor. Methylene blue is very far from being the general acceptor it was once thought to be; 7 of the enzymes in the table do not react with it, and 6 more react only slowly, although it reacts very rapidly with free flavins.

PMS, an azine dye, like methylene blue, has attracted attention as an acceptor that works in cases where others will not, *e.g.* with succinate dehydrogenase, or that works more rapidly than some others. Its reduced form will reduce many acceptors non-enzymically; it is therefore often added as an intermediate catalyst between flavoproteins and other acceptors, for a small amount of phenazine frequently accelerates the reduction of DCIP, ferricyanide, O_2 and even cytochrome *c* by enzymes that cannot reduce them directly[11].

Even this acceptor, however, is not reduced by some flavoproteins, and the incomplete data available show no parallelism in specificity with any of the other acceptors.

Nitrate

This case is different from the others in that nitrate is not an acceptor with free flavins, but does act with certain flavoproteins, suggesting that these enzymes contain a site that activates the nitrate. The nitrate-reducing flavoproteins include xanthine oxidase, aldehyde oxidase of liver (EC 1.2.3.1), and the three nitrate reductases (EC 1.6.6.1, 1.6.6.2 and 1.6.6.3).

It is suggestive that at least three of these (xanthine and aldehyde oxidases and NADPH nitrate reductase) have been shown to contain molybdenum. It seems very probable that this is the site of the nitrate reduction, for SPENCE[44] has very recently shown that a mixture of reduced FMN, Mo(V) and tartrate reduces nitrate non-enzymically in acid solution. It would be interesting to know whether deflavo xanthine oxidase retains its power of reducing nitrate, since it still contains the molybdenum.

POSSIBLE CORRELATIONS WITH OTHER PROPERTIES

There are many special properties that distinguish the various flavoproteins from one another, and it is of interest to consider whether any of these can be correlated with the specificity pattern towards acceptors. Some of these properties are shown in Table II for the enzymes of Table I.

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TABLE II

SOME PROPERTIES OF FLAVOPROTEINS

These data may need revision.

EC No.	*Enzyme*	*Mol. wt.*	*Flavin*	*Flavin groups per molecule*	*Fluorescence*	*Metal*	*Metal atoms per flavin*	*Catalytic mechanism*	*Semiquinone type*
1.1.3.4	Glucose oxidase	186 000	FAD	2	o	o	—	$F \rightleftharpoons FH_2$	Blue / Red
1.1.99.8	Glucose dehydrogenase	100 000		1					
1.1.3.1	Glycollate oxidase	140 000	FMN	2	o	o	—		Red
1.1.3.2	L-Lactate oxidase (or oxygenase) (B)	260 000	FMN	2		o	—		Red
1.1.2.3	L-Lactate dehydrogenase (Y)	160 000	FMN	2		Haem	1	$F \rightarrow FH_2$ / $F \leftarrow FH \leftarrow FH_2$	
1.1.2.4	D-Lactate dehydrogenase (Y)		FAD	1		Zn	3		
1.1.99.6	D-2-Hydroxyacid dehydrogenase	100 000	FAD		o	M?			
1.4.3.2	L-Amino-acid oxidase (SV)	140 000	FAD	2		o	—	$F \rightleftharpoons FH_2$	Red
1.4.3.3	D-Amino-acid oxidase (K)	50 000	FAD	1	+	o	—	$F \rightleftharpoons FH_2$	Red
1.4.3.1	D-Aspartate oxidase (K)								
1.2.3.2	Xanthine oxidase	280 000	FAD	2	o	Mo / Fe	1 / 4		
1.3.3.1	Dihydro-orotate dehydrogenase	62 000	FAD / FMN	1 / 1	+	Fe	1		
1.5.99.1	Nicotine dehydrogenase		FMN			M			
1.6.5.1	Quinone reductase		FMN			o	—		
1.11.1.1	NAD^+ peroxidase		FAD		o	o			
1.6.99.5	NAD(P)H dehydrogenase		FMN						
1.6.2.3	NADPH cytochrome *c* reductase (LM)	89 000	FAD	2		o	—	$FH \rightleftharpoons FH_2$	Blue
1.6.2.2	NADH cytochrome b_5 reductase (LM)	40 000	FAD	1	o	o	—	$F \rightarrow FH_2$ / $F \leftarrow FH \leftarrow FH_2$	Blue
1.3.99.1	Succinate dehydrogenase	150 000–200 000	FAD	1		Fe	4 or 8		
1.6.4.3	Lipoamide dehydrogenase	100 000	FAD	2	+++	o	—		Special

Nature and number of flavin groups

The fact that some flavoproteins contain FMN and others FAD seems not to be of great significance for their catalytic properties. Indeed it might be said that the reacting group of all is FMN, and the additional adenylic acid group attached to it in FAD affects the binding affinity of the flavin to the protein rather than the catalytic mechanism. It is not uncommon to find that after the natural flavin group has been removed the activity can be restored by adding either FMN or FAD to the apoenzyme, although more of the unnatural one may be required because of its smaller affinity. There seems to be no evidence that the acceptor specificity is determined by the nature of the flavin group. Of the enzymes in Table I, those that have FAD appear to have no special property that differentiates them from the FMN enzymes.

Of the flavoproteins for which data are available, about one-third have one flavin group per molecule and about two-thirds have two flavin groups. There is a tendency for the molecular weight per flavin group to lie within the range 70000 ± 25000, so that the actual molecular weights of flavoproteins with two flavin groups are higher than those with one. This suggests that there is only one flavin per peptide chain, and that the proteins which carry two are composed of two sub-units. Indeed lipoamide dehydrogenase has been shown[45] to dissociate reversibly into two, and the monomeric form is even more active with DCIP as acceptor than the dimer, although it no longer uses oxidized lipoate. A special case is dihydroorotate dehydrogenase, which has two different flavin groups (one FMN and one FAD), possibly attached to the same peptide chain.

It has been suggested that where the catalytic mechanism involves the half-reduced (semiquinone) form of the flavoprotein the presence of two flavin groups in the molecule is important for the reduction of those acceptors that require two reducing equivalents, since each of the two half-reduced groups will contribute one equivalent. For example, such a theory has been proposed for L-amino-acid oxidase by WELLNER AND MEISTER[46]. PALMER AND MASSEY[47], however, have demonstrated that this mechanism is incorrect, and they consider that the possession of two flavin groups in a flavoprotein molecule has no more significance than the possession of two independent active centres by many pyridine-nucleotide-using dehydrogenases. In any case, since the majority of flavoproteins have two flavin groups, this cannot explain the differences in acceptor specificity between them.

Metal atoms

A number of flavoprotein enzymes contain one or more metal atoms, usually iron, although at least as many have been shown to be metal-free. The non-haem iron atoms are often associated with an equal number of labile sulphur groups in the protein. The oxidation and reduction of the metal atoms during catalysis may be followed by changes in electron spin resonance (ESR) signals. The free radical semiquinone forms of the flavin groups also give ESR signals, but these can usually be distinguished from those due to metals.

At one time it was thought that the metal was necessary to enable the reduced flavin (a 'two-electron' donor) to reduce 'one-electron' acceptors, such as ferricyanide or cytochrome, but was not necessary for the reduction of 'two-electron' acceptors. The metal atoms could take up one equivalent at a time and pass it on to a one-electron acceptor, and removal of the metal was alleged to prevent reaction with such

acceptors. The evidence, however, has proved to be invalid. In some cases the metal was found to be merely an impurity, not part of the enzyme; the method used to remove the metal did not in fact remove it; in the main case studied, cytochrome *c* was not being reduced by the enzyme at all, but by the superoxide radical formed from oxygen; ferricyanide and some other 'one-electron' acceptors are readily reduced by several metal-free flavoproteins; the cytochrome reductases are metal-free; in cytochrome *c* reductase (EC 1.6.2.3) it has been shown that 'the flavin . . . stubbornly chooses to utilize exactly the same mechanism for reduction of both one- and two-electron acceptors'[22]; and finally there would seem to be little theoretical justification for distinguishing between one- and two-electron acceptors, since all the 'two-electron' acceptors mentioned here can readily be reduced in two one-electron steps, with the intermediate formation of a free radical, usually a semiquinone. In fact, there seems to be no pattern of acceptor specificity characteristic of the metal-containing flavoproteins.

Mechanism of catalysis

While catalysis by all the flavoproteins depends on the reduction and oxidation of their flavin groups, it is not always the fully oxidized and the fully reduced forms that are solely involved. Studies of absorption spectra and ESR signals, using stopped-flow methods, have shown that half-reduced forms of various kinds often play an essential part. But as in all research depending on the interpretation of spectra, there have been some uncertainties about the nature of the compounds, and spectra that had been attributed to semiquinones are now ascribed to other forms, such as charge-transfer complexes and addition compounds.

Three main types of mechanism may be distinguished. The first depends on an oscillation between the fully oxidized and the fully reduced forms of the flavin ($F \rightleftharpoons FH_2$), represented by glucose oxidase[48]. Here the substrate reduces the F form directly to the FH_2 form, without the formation of a free radical semiquinone as an intermediate, and the acceptor oxidizes the FH_2 form directly to F. Although the semiquinone is not involved in the catalysis, it is produced in high yield by reduction with dithionite instead of glucose, with the appearance of a semiquinone spectrum and an ESR signal. Dithionite will convert many flavoproteins into their semiquinones, whether this form is involved in the catalysis or not, and NADH has a strong tendency to do the same. It is remarkable, however, that the semiquinone form of glucose oxidase is catalytically quite unreactive and cannot be fully reduced by substrate[49]. This has been found also with the D- and L-amino-acid oxidases; here what was at first believed to be a semiquinone form produced by the substrate was later said to be a charge transfer complex of the FH_2 form with the oxidized substrate[48].

In a variant of this type of mechanism, represented by cytochrome b_5 reductase, F appears to be reduced to FH_2 in one step by the substrate, but the oxidation by acceptor goes in two steps as $FH_2 \rightarrow FH \rightarrow F$, where FH denotes a half-reduced form.

In the second type, exemplified by lipoamide dehydrogenase, the oscillation is between the oxidized and half-reduced forms ($F \rightleftharpoons FH$) and the FH_2 form is not involved in the catalytic cycle.

In the third type, represented by cytochrome *c* reductase (EC 1.6.2.3), the F form is not involved in the catalysis and the cycle involves the half and fully reduced forms ($FH \rightleftharpoons FH_2$). The FH_2 form is oxidized to the FH form by oxygen,

ferricyanide, cytochrome *c*, menadione, or DCIP, but the FH form is not oxidized further by these acceptors. Thus the acceptor specificities of the fully reduced and half reduced forms of the flavin are different[22].

The abbreviation FH is used here without any implication that the half reduced form produced by substrates (as distinct from dithionite) is a free radical semiquinone; in fact the absence of a free radical ESR signal indicates that it is not, and its nature is still uncertain. MASSEY *et al.*[48] state that 'in contrast to the metalloflavoproteins which all give high yields of free radicals on substrate reduction, metal-free flavoproteins are remarkably consistent in not yielding free radicals on addition of substrates'.

It seems likely that, since the type of mechanism must be partly determined by the relative acceptor specificities of the FH and FH_2 forms, there might well be some correlation between the type and the acceptor specificity of the enzyme, but unfortunately the present data are too few to reveal it.

Other groups

In certain cases there is evidence that, in addition to flavins and metals, there are other groups in the apoenzyme that undergo oxidation and reduction during the catalysis. Particularly in the cases of lipoamide dehydrogenase and glutathione reductase (EC 1.6.4.2) an –SS– group in the protein is oxidised and reduced at the same time as the flavin, and mechanisms have been proposed[48] involving the formation of complexes of half-reduced flavin and thiol groups. It may be more than a coincidence that both these enzymes are concerned with the oxidation of thiol compounds.

It has been suggested from time to time that some flavoproteins contain chromophoric groups other than flavins that undergo oxidation and reduction, but if so their nature is unknown.

Fluorescence

In the oxidized form, free flavins are strongly fluorescent, but while some flavoproteins are also fluorescent, others are not; the very incomplete data available suggest that the majority may be non-fluorescent. The suppression of the flavin fluorescence is usually regarded as an indication that in most cases the protein is combined with the isoalloxazine ring itself, whereas in lipoamide dehydrogenase and one or two other cases this part of the molecule is not directly combined. If so, it is all the more remarkable that the flavin group of lipoamide dehydrogenase is unable to react with a number of acceptors that react very rapidly with the flavin when free. It is possible, however, that the ring becomes bound to the protein when it is reduced; this would not be revealed by the fluorescence, which is confined to the oxidized form. There is no obvious correlation between fluorescence and acceptor specificity.

Redox potential

The various acceptors lie at different points on the redox scale, and this fact must be taken into account in comparing their behaviour towards flavoproteins. From an equilibrium point of view, a flavoprotein would be expected to reduce any acceptor that lies well above it on the redox scale; if it does not, it is because the catalytic mechanism of the enzyme does not permit it. On the other hand, acceptors lying well below the flavoprotein on the scale could not be expected to be reduced by it, whatever the mechanism, except to a very small extent.

TABLE III

REDOX POTENTIALS OF FLAVINS AND ACCEPTORS

E'_0 denotes the redox potential (with respect to the normal hydrogen electrode) of a solution containing equal concentrations of the reduced and oxidized forms.

Substance	E'_0 (*V*)	*pH*	*Temp.*
Oxygen	+0.8	7.6	20°
Ferricyanide	+0.43	7.6	20°
Cytochrome *c*	+0.27	7.4	20°
Benzoquinone	+0.24	7.6	20°
DCIP	+0.16	7.6	20°
Phenazine methosulphate	+0.08	7.0	30°
Methylene blue	0.0	7.4	20°
NADPH dehydrogenase	−0.07	7.0	20°
	−0.12	7.0	30°
FAD	−0.214	7.6	20°
Ascorbate	+0.04	7.6	20°
NAD^+	−0.34	7.6	20°

While the redox potentials of most acceptors are known with fair accuracy (Table III), there are unfortunately very few reliable values for flavoproteins, and the measurements obtained by different authors differ widely. Probably the best measurement is that of VESTLING[50] on NADPH dehydrogenase (EC 1.6.99.1). This is appreciably higher than the same flavin in the free state; this is a consequence of the fact that the affinity of the apoenzyme for the reduced flavin is considerably higher than its affinity for the oxidized form. The few data available suggest that this may be rather a general effect, and we may guess that most flavoproteins lie appreciably higher than the flavins on the scale.

At first sight this might seem to offer a possible explanation for the inhibition by the protein of the reaction of the flavin with certain acceptors, simply by raising the potential of the flavin to a point above that of the acceptor. But this cannot account for the kind of specificity pattern seen in Table I, for if it were true, the acceptors would cease to act one by one in the order of their potentials as the flavoprotein potential becomes more positive, beginning with methylene blue and ending with ferricyanide and oxygen. This order would be the same for all flavoproteins.

Table I shows that the facts are quite otherwise, and there is no correlation between the redox potentials of the acceptors and their reactivity with the flavoproteins. In many cases acceptors with negative potentials are reduced but more positive ones are not, and in general their behaviour seems to bear no relation to their position on the redox scale. If redox potential plays any part in determining acceptor specificity, it can only be a minor one.

Affinity for acceptors

The question arises whether differences in the affinities of flavoproteins for different acceptors play any significant part in determining the specificity. There is an almost complete lack of information about the actual affinities for acceptors. There are a few scattered values for Michaelis constants of acceptors in the literature, and a number have been measured in this laboratory (with assistance from Mr. P. Kenworthy); but such constants do not necessarily represent affinities, and of

course Michaelis constants can only be obtained for acceptors that react with a significant rate. As far as they go, the results suggest that Table I would not appear very different if high concentrations of the acceptors had been used throughout, but it is not possible to be definite on the point.

CONCLUSION

The protein part of a flavoprotein enzyme has several important functions. It provides the substrate-binding site which is responsible for the activation of the substrate and the high substrate specificity of the enzyme. In many cases it carries metal atoms as part of the enzyme structure; these may play a special part in the catalytic mechanism, enabling the enzyme to react with substances that do not react with free flavins, *e.g.* the reduction of nitrate by molybdenum atoms. It acts as a selective inhibitor of the reaction of the flavin with particular acceptors with which the flavin rapidly reacts when free; it thereby creates a pattern of acceptor specificity, imposed on the flavin. It appears that each protein creates a different pattern of specificity for the flavin; this may be of great importance in determining the biological function of the enzyme. It has a stabilizing effect on the semiquinone and other half-reduced forms of the flavin, which can readily be observed in flavoproteins, but are much more difficult to see with free flavins.

The substrate specificity of flavoproteins has been studied in considerable detail, but their acceptor specificity has been comparatively neglected. The effects, however, are of great interest; the differences between different flavoproteins are remarkable, and it seems difficult to explain them by the known properties of the individual proteins. A plea is made for a more intensive study of the acceptor specificity of flavoproteins, in the belief that it will throw valuable light on their mode of action.

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