The influence of oxidation–reduction state on the kinetic stability of pig kidney general acyl-CoA dehydrogenase and other flavoproteins

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Pig kidney general acyl-CoA dehydrogenase is markedly stabilized against loss of flavin and activity in 7.3 M-urea or at 60°C upon reduction with sodium dithionite or octanoyl-CoA. Electron transferring flavoprotein is similarly stabilized, whereas egg white riboflavin-binding protein loses flavin more readily on reduction. These and other data support the anticipated correlation between the kinetic stability of the holoproteins and the oxidation–reduction potential of their bound flavins.

One of the interesting aspects of flavoprotein research is the modulation of the kinetic and thermodynamic properties of the flavin prosthetic group upon binding to the apoprotein. For example, flavoproteins exhibit a range of redox potentials (E'_0 values from –465 to +149 mV; Walsh, 1979) compared with the corresponding value for free flavin of –208 mV (Draper & Ingraham, 1968). This perturbation reflects differential binding of the oxidized and dihydroflavin forms to the apoprotein: a +100 mV increase in E'_0 value dictates an approx. 2000-fold tighter binding of the reduced flavin species over its oxidized counterpart. The present paper shows that the kinetic stability of several flavoproteins towards loss of flavin is also markedly affected upon reduction of the flavin prosthetic group. These data, together with examples from the literature, suggest that this property may be systematically exploited to stabilize or destabilize proteins at will.

Experimental

Materials

Pig kidney general acyl-CoA dehydrogenase was obtained as described previously (Thorpe, 1981), electron transferring flavoprotein was a gift from Mr. Robert Gorelick, University of Delaware (Gorelick *et al.*, 1982), and egg white riboflavin-binding protein (Farrell *et al.*, 1969) was a gift from Dr. Hal White of this Department. Urea, ultra-

pure, was from Schwarz/Mann Inc. and sodium dithionite (low in iron) was from Fisher. Octanoyl-CoA was supplied by P-L Biochemicals. Activated charcoal (Norit A) was from Sigma.

Methods

The buffer used throughout this work was 50 mM-potassium phosphate, pH 7.6, containing 0.3 mM-EDTA. Flavoprotein concentrations were determined with the following absorption coefficients for bound flavin: acyl-CoA dehydrogenase, $15.4 \text{ mM}^{-1} \cdot \text{cm}^{-1}$ at 446 nm (Thorpe *et al.*, 1979); electron transferring flavoprotein, $13.3 \text{ mM}^{-1} \cdot \text{cm}^{-1}$ at 436 nm (Gorelick *et al.*, 1982); and egg white riboflavin-binding protein, $11 \text{ mM}^{-1} \cdot \text{cm}^{-1}$ at 455 nm (Kosik, 1982). Enzyme assays for the dehydrogenase and electron transferring flavoprotein were performed as in Gorelick *et al.* (1982). Anaerobic incubations were performed under N_2 in small glass vials sealed with serum caps. Buffer solutions were deoxygenated by bubbling N_2 for at least 20 min, and were introduced through the serum caps by using syringe needles. Concentrated solutions of sodium dithionite were prepared in the incubation buffers as described in Williams *et al.* (1979) and diluted to 0.5 mM in the vials prior to the addition of small volumes of the flavoprotein solutions to start the incubation. Samples were removed for enzyme assay, or were rapidly gel-filtered at 4°C on a column (1 cm $\times$ 18 cm) of Sephadex G-25. In the case of riboflavin-binding protein, 1 ml samples (oxidized, or reduced with 0.5 mM-dithionite) were mixed with 10% (v/v) of a charcoal slurry [5% (w/w) activated charcoal in buffer] and were centrifuged in an Eppendorf bench-top centrifuge for 3 min. The clear supernatants were removed

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and excess dithionite was discharged by exposing the solutions to air. Recovery of flavoprotein was assessed spectrophotometrically with a Cary 219 instrument.

Results

Fig. 1 shows the effect of 7.3M-urea on the activity of pig kidney general acyl-CoA dehydrogenase in 50mM-phosphate, pH 7.6, at 25°C. Loss of activity is first-order over at least 4 half-lives ($t_{1/2} = 22$ min) whereas a control sample incubated

Fig. 1. Effect of oxidation-reduction state on the stability of pig kidney general acyl-CoA dehydrogenase. General acyl-CoA dehydrogenase ($2.59 \mu\text{M}$ enzyme flavin in 1 ml of 50mM-phosphate buffer, pH 7.6, 25°C) was incubated alone ($\square$), with 7.3M-urea ($\bullet$), or with 7.3M-urea in the presence of 0.5mM-sodium dithionite (Δ , see under 'Methods'), or 100 μM -octanoyl-CoA ($\circ$). Samples were withdrawn and assayed immediately at the times indicated.

in the absence of urea remains stable over this time interval (Fig. 1). Inactivation is associated with a release of flavin from the enzyme. Thus when the dehydrogenase is diluted into 7.3M-urea, the characteristic visible spectrum of the flavoprotein is replaced by that of free FAD in urea in a reaction showing first-order kinetics with a half-time of 23 min (results not shown). Apoprotein prepared by urea treatment and subsequent dialysis against phosphate buffer, pH 7.6, is irreversibly denatured and can no longer bind FAD (Mayer, 1981). Fig. 1 also shows the marked kinetic stabilization against denaturation which reduction affords. This is observed not only upon the addition of octanoyl-CoA, a tightly bound substrate of this dehydrogenase (Thorpe *et al.*, 1981), but even better protection is obtained on chemical reduction of the flavin prosthetic group with sodium dithionite (97% activity remaining after 230 min; Table 1). This stabilization is not restricted to the use of urea as a denaturant. Table 1 shows that a marked protection against inactivation is also observed when the reduced enzyme is incubated at 60°C in 50mM-phosphate buffer, pH 7.6.

Table 1 also shows the stability of two other flavoproteins as a function of their redox state. Oxidized pig kidney electron transferring flavoprotein releases FAD in 3.4M-urea in a reaction which is first-order over 4 half-lives (followed spectrophotometrically, $t_{1/2} = 18$ min). Gel filtration of the oxidized protein after 100 min (>5 half-lives) incubation in urea led to the recovery of 8% holoenzyme. The apoprotein in these fractions is unable to rebind flavin. In contrast, comparable treatment of the dihydroflavin species enabled 58% recovery of holoenzyme to be realized. Release of flavin from electron transferring flavoprotein cannot be assessed enzymically in the standard assay system where it serves as a mediator between substrate-reduced acyl-CoA dehydro-

Table 1. Stability of flavoproteins in their oxidized and dihydroflavin forms. Samples were treated as indicated in 50mM-potassium phosphate buffer, pH 7.6, containing 0.3mM-EDTA.

Flavoprotein	E'_0 (mV)	Treatment	Oxidation-reduction state	Activity or flavin retained (%)
General acyl-CoA dehydrogenase	-128*	7.3M-urea, 230 min, 25°C	Ox.	0
			Red.	97
General acyl-CoA dehydrogenase	-128	60°C, 11 min	Ox.	2
			Red.	89
Electron transferring flavoprotein	-23†	3.4M-urea, 100 min	Ox.	8
			Red.	58
Egg white riboflavin-binding protein	-330‡	Charcoal slurry	Ox.	80
			Red.	12

* Feinberg & Gustafson (1982).

† Husain *et al.*, 1984.

‡ Spencer (1978).

genase and 2,6-dichlorophenolindophenol (Gorelick & *et al.*, 1982). This is because the dye undergoes reduction catalysed by apo-(electron transferring flavoprotein) (R. J. Gorelick, S. M. Lau & C. Thorpe, unpublished results). In the absence of electron transferring flavoprotein, reduction of the dye by the acyl-CoA dehydrogenase is extremely slow (Thorpe *et al.*, 1979).

In contrast to these examples, egg white ribo-flavin-binding protein loses flavin much more readily upon reduction (Table 1) and it has been reported to bind dihydriboflavin much more weakly than the oxidized form (Spencer, 1978; Blankenhorn, 1980). Loss of flavin from the holo-enzyme was induced by using a slurry of activated charcoal to bind any free riboflavin (Mayer & Thorpe, 1981).

Discussion

The present paper shows that the kinetic stability of flavoproteins is markedly influenced by the oxidation–reduction state of their flavin prosthetic groups, and several examples from the literature support this correlation. Lipoamide dehydrogenase is very stable in 7.5M-urea in its oxidized form, but rapidly loses flavin upon reduction with dithionite, NADH, or dihydro-lipoic acid (Massey *et al.*, 1962). In addition to FAD, lipoamide dehydrogenase contains a redox-active disulphide bridge and thus can accept a total of 4 reducing equivalents [$E'_0 = -280\text{ mV}$ (E/EH₂); $E'_0 = -346\text{ mV}$ (EH₂/EH₄); Matthews & Williams, 1976]. It could be argued that labilization of the enzyme is the result of reduction of the active centre disulphide rather than the flavin prosthetic group. However a derivative of lipoamide dehydrogenase in which the disulphide bridge is reduced and monoalkylated is similarly sensitive to loss of flavin and denaturation upon reduction of the enzyme with either NADH or dithionite (Thorpe & Williams, 1976). Veeger and coworkers (Voordouw *et al.*, 1982) have observed that pyridine nucleotide transhydrogenase from *Azotobacter vinelandii* is considerably less stable toward thermal and guanidine hydrochloride denaturation when reduced by NADH or sodium dithionite. Indeed, they prepared a partially reconstitutable apoprotein by gel filtration of the transhydrogenase in the presence of NADH. In view of the nicotinamide nucleotide substrates of this enzyme, the redox potential of the bound flavin would be expected to be in the -300 mV range. L-Amino acid oxidase from snake venom is stabilized by reduction with L-leucine (Singer & Kearney, 1950; Wellner & Meister, 1960). Increased thermal stability was also observed on reduction with sodium dithionite (Wellner & Meister, 1960). The

two-electron redox potential for L-amino acid oxidase has recently been determined as -115 mV (Stankovich & Fox, 1984) a value considerably more positive than that of free FAD.

In all these instances, those flavoproteins with oxidation–reduction potentials more positive than free flavin are more stable to loss of flavin in their reduced states. Conversely, those proteins having E'_0 values more negative than about -208 mV are more labile upon reduction. One plausible explanation for this correlation stems from the fact that differences in the dissociation constants for flavin binding are probably dominated by changes in the dissociation rate constant, k_{off} (Becvar & Palmer, 1982):

Thus, for a flavoprotein having an E'_0 value more positive than free flavin, the off constants for reduced flavin would be expected to be correspondingly smaller than for dissociation of flavin from the oxidized protein. Loss of flavin may or may not entail irreversible loss of reconstitutability under the conditions employed. Nevertheless, apoproteins are, in general, markedly less stable than their corresponding holoenzyme forms (Husain & Massey, 1979). In the present case, urea treatment causes irreversible denaturation of general acyl-CoA dehydrogenase and electron transferring flavoprotein, although reconstitutable apoprotein may be obtained by an acid (NH₄)₂SO₄ precipitation procedure (Mayer & Thorpe, 1981).

This paper has presented a correlation between the kinetic stability of flavoproteins towards loss of flavin and the oxidation–reduction potentials of their bound prosthetic groups. A more general appreciation of this phenomenon might facilitate the purification, storage, and study of other flavoproteins.

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References

- Becvar, J. & Palmer, G. (1982) *J. Biol. Chem.* **257**, 5607–5617
- Blankenhorn, G. (1980) in *Flavins and Flavoproteins* (Yagi, K. & Yamano, T.), pp. 405–411, Japanese Scientific Societies Press, Tokyo
- Draper, F. D. & Ingraham, L. L. (1968) *Arch. Biochem. Biophys.* **125**, 802–808
- Farrell, H. M., Jr., Mallette, M. F., Buss, E. G. & Clagett, C. O. (1969) *Biochim. Biophys. Acta* **194**, 433–442
- Feinberg, B. A. & Gustafson, W. G. (1982) in *Flavins and Flavoproteins* (Massey, V. & Williams, C. H., eds.), pp. 632–636, Elsevier/North-Holland, New York

- Gorelick, R. J., Mizzer, J. P. & Thorpe, C. (1982) *Biochemistry* **21**, 6936-6942
- Husain, M. & Massey, V. (1979) *Methods Enzymol.* **53**, 429-437
- Husain, M., Stankovich, M. T. & Fox, B. G. (1984) *Biochem. J.* **219**, 1043-1047
- Kosik, A. (1982) *Eur. J. Biochem.* **121**, 395-400
- Massey, V., Hofmann, T. & Palmer, G. (1962) *J. Biol. Chem.* **237**, 3820-3828
- Matthews, R. G. & Williams, C. H., Jr. (1976) *J. Biol. Chem.* **251**, 3956-3964
- Mayer, E. J. (1981) Ph.D. Thesis, University of Delaware
- Mayer, E. J. & Thorpe, C. (1981) *Anal. Biochem.* **116**, 227-229
- Singer, T. P. & Kearney, E. B. (1950) *Arch. Biochem. Biophys.* **29**, 190-209
- Spencer, R. (1978) Ph.D. Thesis, Massachusetts Institute of Science and Technology
- Stankovich, M. T. & Fox, B. G. (1984) *Biochim. Biophys. Acta*, in the press
- Thorpe, C. (1981) *Methods Enzymol.* **71**, 366-374
- Thorpe, C. & Williams, C. H., Jr. (1976) *J. Biol. Chem.* **251**, 3553-3557
- Thorpe, C., Matthews, R. G. & Williams, C. H., Jr. (1979) *Biochemistry* **18**, 331-337
- Thorpe, C., Ciardelli, T. L., Stewart, C. J. & Wieland, Th. (1981) *Eur. J. Biochem.* **118**, 279-282
- Voordouw, G., de Haard, H., Timmermans, J. A. M., Veeger, C. & Zabel, P. (1982) *Eur. J. Biochem.* **127**, 267-274
- Walsh, C. (1979) *Enzymatic Reaction Mechanisms*, pp. 358-405, Freeman and Co., San Francisco, CA
- Wellner, D. & Meister, A. (1960) *J. Biol. Chem.* **235**, 2013-2018
- Williams, C. H., Jr., Arscott, L. D., Matthews, R. G., Thorpe, C. & Wilkinson, K. D. (1979) *Methods Enzymol.* **62**, 185-198