[Nitrotyrosyl]Cytochrome *c*

STUDIES OF THE EFFECT OF IRON BINDING, PROTEIN DENATURANTS AND OXIDATION-REDUCTION POTENTIALS

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Static measurements of the reaction of ligand binding were done by conventional spectrophotometry. The ligand-binding reactions with nitrated cytochrome *c* were performed with imidazole, iminazole, CO and NO. The stoichiometry was found to be 1:1, and the stability constants for the complexes formed between the nitrated cytochrome *c* and the ligands are: $2.58 \times 10^4 \text{ M}^{-1}$ (imidazole); $1.01 \times 10^2 \text{ M}^{-1}$ (iminazole); $3.6 \times 10^4 \text{ M}^{-1}$ (CO); $2.74 \times 10^4 \text{ M}^{-1}$ (NO). It was found that the electrometric potentials at pH 7.0 and 25°C of [aminotyrosyl]cytochrome *c* are $E'_{\text{form II}} = 0.115 \text{ V}$ and $E'_{\text{form I}} = 0.260 \text{ V}$, where forms I and II are two species of protein co-existing in the protein solution. The isoelectric point for the oxidized form of [nitrotyrosyl]cytochrome *c* was 10.05, at 4°C.

Several agents such as pH and iron ligands displace the haem ligands of [nitrotyrosyl]cytochrome *c*, transforming the protein from a mixed-spin state to a fully high-spin or low-spin configuration (Sokolovsky *et al.*, 1970). The modified protein was interpreted by Schejter *et al.* (1970) as consisting of two species, derived from the nitration of one of the two positions ortho to the tyrosine-67 hydroxyl group, arising from the fact that the residue is unable to rotate. They refer to these species as form I and form II.

The purpose of the experiments reported here is to investigate some of the physicochemical properties of cytochrome *c* with tyrosine-67 modified, and analyse the significance of the role played by tyrosine-67 in the structure of native cytochrome *c*.

Materials and Methods

Native cytochrome *c* (type III), guanidine and imidazole were obtained from Sigma (London) Chemical Co., Kingston-upon-Thames, Surrey, U.K., and were used without further purification. Iminazole was purchased from BDH, Poole, Dorset, U.K. The concentration of cytochrome *c* solutions was estimated by using a millimolar extinction coefficient of $27.7 \text{ litre} \cdot \text{mmol}^{-1} \cdot \text{cm}^{-1}$ at 550 nm for the reduced protein (Margoliash, 1954).

[Nitrotyrosyl]cytochrome *c* was prepared as described by Sokolovsky *et al.* (1970), and the concentration was estimated at 530 nm by using a millimolar extinction coefficient of $9.8 \text{ litre} \cdot \text{mmol}^{-1} \cdot \text{cm}^{-1}$,

at pH 7.0 (Schejter *et al.*, 1970), after addition of solid ferricyanide.

Determination of the isoelectric point was carried out at 4°C in an LKB 8101 isoelectric-focusing apparatus (110 ml column). Approx. 4 mg of protein was applied to the column and focused in the pH range 9–11 as described by Karlsson & Ohman (1972). The pH values of the solutions were measured at 4°C by using an EIL pH-meter 23A, fitted with a Russell microelectrode CMT 21.

Spectrophotometric observations were carried out with a Unicam SP.700A instrument, with a thermostatically controlled compartment. The temperature of the cell compartment was kept constant by water circulation by using a thermostatic bath (Grant Instruments, Cambridge, U.K.). Anaerobic spectrophotometry was carried out in special cuvettes fitted with a vaccine cap. For spectrophotometric titrations, samples of the ligand were added with an Agla micrometer syringe. The volume of the protein solution was measured by weighing the cuvette after the completion of the experiment.

Spectrophotometric observations of the disappearance of the 695 nm band of [nitrotyrosyl]ferricytochrome *c* with increasing temperature were analysed by using the assumptions described by Schejter & George (1964). Results were analysed as a displacement of equilibrium between two conformational isomers, one containing the 695 nm band ('cold' conformation) and the other lacking this band ('hot' conformation). The molar extinction coefficient at 695 nm at pH 4.5 for the [nitrotyrosyl]ferricytochrome *c* [$(\epsilon_{695})_c$] was calculated to be $600 \text{ litre} \cdot \text{mol}^{-1} \cdot \text{cm}^{-1}$ at 10°C for the 'cold' conformation and $439 \text{ litre} \cdot \text{mol}^{-1} \cdot \text{cm}^{-1}$ in 6 M-guanidine

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at the same temperature for the 'hot' conformation [$(\epsilon_{695})_h$].

Gases were supplied by British Oxygen Company, Deer Park Road, London S.W.19, U.K., except for NO, which was supplied by Matheson Gas Products, East Rutherford, NJ, U.S.A. N_2 and CO were stored in glass vessels under alkaline dithionite-reduced anthraquinone solution.

Measurements of electrometric potentials were carried out under anaerobic conditions in a jacketed glass vessel, adequate for titration of a 15 ml sample. All measurements were made at $25 \pm 1^\circ\text{C}$. Potassium ferricyanide (obtained from Hopkin and Williams, Chadwell Heath, Essex, U.K.) was used as oxidant and was added to the vessel with an Agla micrometer syringe. Stirring was effected with a Teflon-coated magnetic bar and an immersible magnetic stirrer (Rank Brothers, Cambridge, Cambs., U.K.). The reaction medium contained about a $3.0 \mu\text{M}$ solution of buffered [nitrotyrosyl]cytochrome c. Constant H^+ ion concentration during the electrometric titration was maintained by means of sodium acetate buffer (pH 5.5–5.8), sodium phosphate buffer (pH 6.0–7.8), Tris/HCl buffer (pH 8.0–9.0) and glycine/NaOH buffer (pH 9.2–9.5). The total ionic strength of the reaction medium was kept below 0.006 mol/litre to remain in the Debye-Huckel region (any residual junction potential in the region of very low ionic strength is either insignificantly small or is virtually constant). The protein was fully reduced before the titration by adding a freshly prepared solution of dithionite (10 mg/ml) (obtained

from Hardman & Holden, Manchester, U.K.). Then $10 \mu\text{l}$ of the mediator *NNN'*-tetramethyl-*p*-phenylenediamine was added to give a concentration of $8.0 \mu\text{M}$ (final concentration).

Potentiometric measurements were made between a platinum electrode (Radiometer P101) and a calomel electrode (Radiometer K 401), by using a Radiometer pH-meter 51B. Equilibrium potential values (E_h) were obtained at 10 min intervals after addition of the titration agent. Titration was monitored by direct-vision spectroscopy, by using a Hartridge Reversion Spectroscope (Beck, London, U.K.).

O_2 -free N_2 was further purified by passage through two wash bottles fitted with sintered-glass discs and filled with Cr^{2+} solution ($CrCl_2$) over granulated zinc. Before an experiment, the titration vessel and contents were thoroughly flushed free of O_2 .

Results

Effect of guanidine hydrochloride and temperature on the 695 and 612 nm band

Fig. 1 represents the spectra of [nitrotyrosyl]-ferricytochrome c recorded at 10° and 45°C in the region of 612 and 695 nm. Similar behaviour is observed on addition of guanidine hydrochloride to [nitrotyrosyl]ferricytochrome c, as shown in Fig. 2, as the high-spin 620 nm band appears at full strength on the type I denatured conformation. Fig. 3 shows the effect of temperature on the E_{695} of [nitrotyrosyl]-ferricytochrome c. The plot of the log of absorbance

Fig. 1. Spectra of [nitrotyrosyl]ferricytochrome c at 10° and 45°C recorded in the region of 612 and 695 nm

(a) Spectra recorded at two temperatures in the region of 612 nm with 0.344 mM [nitrotyrosyl]ferricytochrome c. (b) Spectra recorded at two temperatures in the region of 695 nm with 1.456 mM [nitrotyrosyl]cytochrome c. The buffer used was 0.1 M -sodium acetate, pH 4.5.

Fig. 2. Effect of guanidine on the 612 and 695 nm band of [nitrotyrosyl]ferricytochrome *c*

(a) Spectra recorded in the region of 612 nm. (b) Spectra recorded in the region of 695 nm. [Nitrotyrosyl]ferricytochrome *c* was used in this experiment at 0.580 mM. The buffer used was 0.1 M-sodium acetate, pH 4.5. ○, Native protein; ●, protein in 4.3 M-guanidine. The temperature was 10°C.

Fig. 3. Effect of temperature on the absorbance of the 695 nm band of [nitrotyrosyl]ferricytochrome *c*

The protein concentration was 1.456 mM. The experiment was performed in 0.1 M-sodium acetate buffer, pH 4.5. The temperature was gradually increased from 10° to 60°C.

Fig. 4. Van't Hoff plot for the data of Fig. 3

Plot of the log K_{obs} versus T⁻¹ for the reaction [nitrotyrosyl]ferricytochrome *c* 'cold' ↔ [nitrotyrosyl]ferricytochrome *c* 'hot', by the equation

$$K_{\text{obs}} = \frac{(E_{695})_t - (E_{695})_c}{(E_{695})_h - (E_{695})_t}$$

of Fig. 3 (see the text for details).

change versus reciprocal of temperature is shown in Fig. 4. The plot is linear for the interval 22–57.5°C, and the thermodynamic parameters evaluated for this temperature-dependent isomerism at 26°C are

Fig. 5. Difference spectra of ferricytochrome *c* and ferricytochrome *c*-imidazole complex

Nitrated and native ferricytochrome *c* (0.112 mM, after mixing) was treated with 1.0 M-imidazole (final concn. after mixing). —, Modified protein; ----, native cytochrome *c*. The experiments were performed in 0.1 M-sodium phosphate buffer, pH 7.0. The reference cell was made 1.0 M with NaCl to give a constant ionic strength.

Fig. 6. Effect of iminazole on the 612 and 695 nm bands

(a) Spectra of [nitrotyrosyl]ferricytochrome *c* (—) and [nitrotyrosyl]ferricytochrome *c*-iminazole complex (----), recorded in the region of 695 nm. (b) Spectra of [nitrotyrosyl]ferricytochrome *c* (—) and [nitrotyrosyl]ferricytochrome *c*-iminazole complex (----) recorded in the region of 612 nm. The protein (0.790 mM, final concn. after mixing) was treated with 0.136 M-iminazole (final concn. after mixing). The experiments were performed in 0.1 M-sodium acetate buffer, pH 4.5. The 695 nm band was recorded in a cell of 2 cm light-path, and the 612 nm band was recorded in a cell of 1 cm path-length.

$\Delta G^0 = 4.58 \pm 0.42 \text{ kJ} \cdot \text{mol}^{-1}$, $\Delta H^0 = 54.46 \pm 1.17 \text{ kJ} \cdot \text{mol}^{-1}$ and $\Delta S^0 = 167.36 \pm 2.09 \text{ kJ} \cdot \text{mol}^{-1} \cdot ^\circ\text{C}^{-1}$.

Effect of imidazole and iminazole

On addition of imidazole to [nitrotyrosyl]ferricytochrome *c* at pH 7.0 a low-spin conformation is obtained, as evidenced by the disappearance of the

612 nm band and enhancement of the 530 nm band (Fig. 5). We observed that the closely related compound iminazole can bind the protein at pH 4.5, and the effects of this ligand on the 695 nm band of the low-spin conformation and the 612 nm band of the high-spin conformation are shown in Fig. 6.

The spectrophotometric titration of [nitrotyrosyl]ferricytochrome *c* with imidazole (pH 7.0) and imin-

Fig. 7. Hill plot of the titration of [nitrotyrosyl]ferricytochrome *c* with imidazole (a) and iminazole (b)

Plot of $\ln(\bar{Y})/\ln(1-\bar{Y})$ versus $\ln[\text{imidazole}]$ or $\ln[\text{iminazole}]$, where $\bar{Y}$ represents the fraction of site-occupied protein. [Nitrotyrosyl]ferricytochrome *c* (0.791 mM) was titrated with 1.22 M solutions of imidazole or iminazole. The imidazole titration (a) was performed in 0.1 M-sodium phosphate buffer, pH 7.0, at 604 nm. The iminazole titration (b) was performed in 0.1 M-sodium acetate buffer, pH 4.5, at 695 nm.

Table 1. Affinity constants for the reaction of [nitrotyrosyl]-cytochrome *c* and cytochrome *c* with ligands

Affinity constants were determined from plots of $\ln(\bar{Y})/\ln(1-\bar{Y})$ versus $\ln(S)$, where $\bar{Y}$ represents the fraction of the site-occupied protein and S represents the concentration of ligand added. The buffers used were sodium acetate buffer, pH 4.5, sodium phosphate buffer, pH 6–7, $\text{Na}_2\text{CO}_3/\text{NaHCO}_3$, pH 10, and ionic strength of 0.1 mol/litre. The values are means of four determinations.

	K_a (M^{-1})	Iron form
[Nitrotyrosyl]cytochrome <i>c</i>		
Imidazole (pH 7)	$2.58 \times 10^4 \pm 0.02 \times 10^4$	Ferric
Iminazole (pH 4.5)	$1.01 \times 10^2 \pm 0.09 \times 10^2$	Ferric
CO (pH 10)	$1.40 \times 10^4 \pm 0.06 \times 10^4$	Ferrous
CO (pH 6)	$2.74 \times 10^4 \pm 0.05 \times 10^4$	Ferrous
NO (pH 10)	$2.74 \times 10^4 \pm 0.05 \times 10^4$	Ferrous
Native cytochrome <i>c</i>		
Imidazole (pH 7)	18.97 ± 0.31	Ferric
Iminazole (pH 4.5)	0.91 ± 0.07	Ferric

azole (pH 4.5) are shown as a Hill plot in Fig. 7. Only unprotonated imidazole as a ligand of cytochrome *c* (Schejter & Aviram, 1969). At pH 7.0, the ratio of protonated imidazole to unprotonated species is 1.06. This value was used to calculate the concentration of unprotonated imidazole and the value of the equilibrium constant in Table 1.

Effect of CO

Fig. 8 illustrates the difference spectra of the [nitrotyrosyl]ferrocycytochrome *c*-CO complex and [nitrotyrosyl]ferrocycytochrome *c* at various pH values and at constant concentration of protein and CO. Fig. 9 illustrates the Hill plot for the spectrophotometric titration of [nitrotyrosyl]ferrocycytochrome *c* with CO solution, at 410 nm. The equilibrium constant calculated from Fig. 9 is listed in Table 1.

Effect of NO

Spectrophotometric titration of [nitrotyrosyl]ferrocycytochrome *c* with NO was performed at alkaline pH at 550 nm. The equilibrium constant estimated from Fig. 10 is listed in Table 1.

Oxidation-reduction potential

Dithionite reduces the nitro group of the tyrosine to the amino group very rapidly (Sokolovsky *et al.*, 1966). Therein, the dithionite-reduced [nitrotyrosyl]cytochrome *c* is called [aminotyrosyl]cytochrome *c*.

The electrometric data were analysed graphically to estimate the potential at 50% oxidation of the protein, as illustrated in Fig. 11, when two values were obtained for each titration, because of the two forms of modified protein that co-exist in solution (form I and form II). The standard reduction potential values

Fig. 8. Difference spectra of [nitrotyrosyl]ferrocyclochrome *c* and [nitrotyrosyl]ferrocyclochrome *c*-CO complex, as function of pH

[Nitrotyrosyl]ferrocyclochrome *c* (17.3 μM) was incubated with saturated CO at room temperature. The protein was reduced in the absence of air with excess of ascorbate. The experiment was carried out in a Thunberg-type cuvette (1 cm light-path). The inset shows an experiment carried out with 8.2 μM -[nitrotyrosyl]ferrocyclochrome *c*. —●—, pH 2.1 in 0.1 M-glycine/HCl buffer; —, pH 4.5 in 0.1 M-sodium acetate buffer; ---, pH 6.0 in 0.1 M-sodium phosphate buffer; -.-.-, pH 10.1 in 0.1 M-glycine/NaOH buffer; ····, pH 7.0 in sodium phosphate buffer.

for [aminotyrosyl]cytochrome *c* was determined to be +115 mV for the larger fraction (form II) and +260 mV for form I, at pH 7 and 25°C.

Fig. 9. Hill plot for the titration of [nitrotyrosyl]ferrocyclochrome *c* with CO

[Nitrotyrosyl]ferrocyclochrome *c* (5.09 μM) was titrated with 1 mM solution of CO. The experiment was carried out in 0.1 M- $\text{Na}_2\text{CO}_3/\text{NaHCO}_3$ buffer, pH 10.0. The protein was reduced with excess of ascorbate. The reaction was observed at 410 nm.

Fig. 10. Hill plot for the titration of [nitrotyrosyl]ferrocyclochrome *c* with NO

Plot of $\ln(Y)/\ln(1-Y)$ versus $\ln(\text{NO})$, where $\bar{Y}$ represents the fraction of site-occupied protein. [Nitrotyrosyl]cytochrome *c* (ferrous form) was titrated with a 2.06 mM solution of NO. The buffer used was 0.1 M- $\text{Na}_2\text{CO}_3/\text{NaHCO}_3$, pH 10.0. The protein was reduced with excess of ascorbate. The reaction was monitored at 550 nm.

The oxidation-reduction potentials at 25°C through the pH range 5.5–9.5 are given in Fig. 12. The potential at 25°C, in the linear portion of the dependence curve varies with pH according to the equation:

$$\frac{\Delta E_0}{\Delta \text{pH}} = \frac{-0.059 \times a}{n} \quad (1)$$

where E_0 represents the standard reduction potential, a the number of H^+ ions released in the oxidation reaction and n the number of electrons involved in the process (Fruton & Simmonds, 1963). The titration performed spectrophotometrically shows clearly that $n = 1$ for the cytochrome *c*-ferricyanide system (Hawkrige & Kuwana, 1973). The values for the slope, pK and a calculated from Fig. 12 are listed in Table 2.

Determination of the isoelectric point

The isoelectric point of the [nitrotyrosyl]cytochrome *c* was determined to be 10.05 at 4°C (Fig. 13). During the electrofocusing experiments, the native molecule is gradually reduced, until it is fully reduced

Fig. 11. A typical potentiometric titration curve obtained for the [aminotyrosyl]cytochrome *c* system

Plot of the equilibrium potential values (E_h) versus $[Fe(CN)_6]^{3-}$. [Aminotyrosyl]ferrocycytochrome *c* ($3.0 \mu M$) was titrated with 1 mM-potassium ferricyanide. The iron and the nitro group of the protein was reduced with excess of dithionite. The buffer used was 0.002M-sodium phosphate, pH 7.0. ●, Potentiometric curve; ○, first-derivative curve. The results yield standard reduction potential of 0.115V for the larger fraction (form II) and 0.260V for the smaller fraction (form I).

at the end of the run. Unlike the native protein, the [nitrotyrosyl]cytochrome *c* is not reduced during the experiment, and its pI value reported here corresponds to the ferric form.

Fig. 12. Observed standard redox potential of [aminotyrosyl]-cytochrome *c* between pH 5.5 and 9.5

The solid line was calculated from the formula:

$$E_0 = E_m + \frac{0.059a}{n} \times \ln \frac{[H^+]}{[H^+] + K}$$

●, Larger fraction (form II) with $E_m = +0.115V$, $a = 4.23$ and $K = 1.58 \times 10^{-8}M$. ○, Smaller fraction (form I) with $E_m = +0.260V$, $a = 3.89$ and $K = 1.58 \times 10^{-8}M$. E_0 represents the observed standard potential. The buffers used were sodium phosphate buffer (pH 6–7.8), Tris/HCl buffer (pH 8–9) and glycine/NaOH buffer (pH 9.2–9.5). Measurements were made at 25°C and an ionic strength of 0.002 mol/litre.

Table 2. Values of the variation of the potential for unit pH decrease ($\Delta E_0/\Delta pH$), number of H^+ released (a) and the pK of ionization in the process, at alkaline pH

Constants were calculated from slope and point of intersection of the plots of E_0 versus pH (eqn. 1). Measurements were made at 25°C and an ionic strength of 0.002 mol/litre. The buffers used were sodium phosphate buffer (pH 6–7.8), Tris/HCl buffer (pH 8.0–9.0) and glycine/NaOH buffer (pH 9.2–9.5). The values for unit pH decrease are means of four determinations.

	$(\Delta E_0/\Delta pH)$	a	pK	
[Aminotyrosyl]cytochrome <i>c</i> , form I	0.229 ± 0.031	3.89	7.8	This work
[Aminotyrosyl]cytochrome <i>c</i> , form II	0.250 ± 0.023	4.23	7.8	This work
Native cytochrome <i>c</i>	0.06	1	7.8	Rodkey & Ball (1950)

Fig. 13. Results of analysis of fractions from the iso-electric-focusing experiment for [nitrotyrosyl]ferricytochrome *c*

Approx. 4mg of protein was applied to the column. Sorbitol was used as stabilizing medium. ●, [Nitrotyrosyl]ferricytochrome *c*; ○, pH of the fraction at 4°C. The results yield an isoelectric point of 10.05 at 4°C.

Discussion

The conformational change of [nitrotyrosyl]cytochrome *c* observed at 695nm on increasing the temperature is accompanied by a more favourable enthalpy change than for the native protein, and an equally high entropy change. The enthalpy change is lowered by about 6.7kJ/mol on nitration of cytochrome *c*.

No haem protein exists in the low-spin form without two strong ligands co-ordinated to the iron (Zerner *et al.*, 1966), and the imidazole and iminazole experiments can be interpreted as the ligand replacing the sixth ligand in the form I configuration of [nitrosyl]cytochrome *c*, and replacing water as the fifth and sixth ligands and pushing the iron into the porphyrin plane in the form II configuration of [nitrotyrosyl]cytochrome *c*. The reaction can be illustrated as

where L represents the unknown fifth ligand, and Im represents the added ligand.

The nitration of cytochrome *c* confers on the protein a marked pH-dependence, which seems to be the result of a weak stability of the protein conformation. The protein is very unstable at low ionic strength. Dialysis against glass-distilled water makes the nitrated protein very insoluble. Further, if the protein is forced to assume a high-spin configuration by either being submitted to a strongly acidic environment or adding guanidine hydrochloride the completely denatured blue-shifted protein is obtained.

The shift of the Soret peak (blue shift) of native cytochrome *c* has been known for a long time. Several workers have observed the shift under a variety of conditions (Theorell & Åkesson, 1941*b*; Boeri *et al.*, 1953; Stellwagen, 1967; Kaminsky & Davison, 1969; Myer, 1968; Wilson, 1970). However, the effect of the blue shift on the colour of the native cytochrome *c* is only to bleach the red colour.

The amount of protein reduced by ascorbate below pH7.0 remains approximately constant, and it seems clear from Fig. 8 that the spectrum of [nitrotyrosyl]ferrocyanochrome *c*-CO complex is also pH-dependent. If the protein is made fully reduced by addition of dithionite or Cr^{2+} , the reaction with CO is improved but the extinction coefficients of the CO compound, at their extinction maxima, are lower than the values obtained for more drastic modifications of cytochrome *c* (see, e.g., Tsou, 1951; Brunori *et al.*, 1973).

The value of *a* corresponds closely to four (Table 2). It can be inferred that the alkaline proton-linked conformational change of [aminotyrosyl]cytochrome *c* involves the liberation of four H^+ ions. The native molecule has one proton linked with the alkaline conformational change, although two protons are believed to be associated with the conformational change taking place at pH values lower than 1.75 (Rodkey & Ball, 1950).

The proton-linked conformational changes at alkaline pH, from studies performed on alkylated cytochrome *c*, have been associated with the lysine-79 residue (Brunori *et al.*, 1973) and the histidine-18 residue (Aviram & Krauss, 1974).

The participation of tyrosine-67 in the alkaline conformation change has been suggested by Salemm *et al.* (1973). The presently available information does not help us to understand this process, but the nitration of tyrosine-67 not only lowers the *pK* of transition of type II to type IV cytochrome *c* (Skov & Williams, 1971), but also increases the apparent number of protons liberated in the alkaline conformation to four and decreases the isoelectric point of cytochrome *c* from 10.6 to 10.05 at 4°C (Fig. 13) (Theorell & Åkesson, 1941*a*).

Fig. 12 shows that both form I and form II con-

figurations of [aminotyrosyl]cytochrome *c* undergo ionization at alkaline pH, followed by a change in conformation to a form with much lower redox potential. The redox potential at pH9.5 for the major fraction (form II configuration) was determined to be -290mV (Fig. 12). This value is similar to the E_0 values obtained for enzyme-digested haemopeptide cytochrome *c* (Minakami *et al.*, 1957; Harbury & Loach, 1960).

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